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Attorney Docket No. CFBF-P04-002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Applicant:	Wagner et al.	Examiner:	P. Gambel
Serial No.:	09/883,642	Art Unit:	1644
Filing Date:	June 18, 2001		
For:	METHOD FOR TREATING AND PREVENTING ATHEROSCLEROSIS		

CERTIFICATE OF MAILING UNDER 37 C.F.R. §1.8(a)

The undersigned hereby certifies that this document is being placed in the United States mail with first-class postage attached, addressed to the Commissioner for Patents, Mail Stop Appeals, on 5/14/04.

Patricia McKenney

Mail Stop Appeal
Commissioner for Patents
P.O. 1450
Alexandria, VA. 22313-1450
ATTENTION: Board of Patent Appeals and Interferences

Sir:

APPELLANT'S BRIEF ON APPEAL

This is an appeal to the Board of Patent Appeals and Interferences (the "Board") from the decision of the Examiner finally rejecting claims 39-52, 61-68, 71-73 and 76-79, and is in furtherance of the Notice of Appeal filed on January 20, 2004, in this application. The appealed claims are as set forth in the attached Appendix. Provision for the payment of fees required for filing this brief, and any required extension of time for filing the brief, is submitted herewith. This brief is submitted in triplicate in accordance with the provisions of 37 C.F.R. §1.192(a).

REAL PARTY IN INTEREST

The real party in interest in this appeal is the CBR Institute for Biomedical Research, Inc., aka The Center for Blood Research, Inc., the assignee of the rights of the inventors in the above-identified patent application. The CBR Institute for Biomedical Research, Inc., is an affiliate of the Harvard Medical School.

RELATED APPEALS AND INTERFERENCES

The present application is a continuation of U.S. Application No. 09/436,076, filed November 8, 1999, which is also currently the subject of an appeal. The outcome of that appeal may have some bearing on the resolution of this appeal. There are no other related appeals or interferences that will directly affect, be directly affected by, or have a bearing on the Board's decision in this appeal.

STATUS OF CLAIMS

The status of the claims in this application is as follows. Claims 39-52, 61-68, 71-73 and 76-79 are pending and are on appeal. Claims 53-60, 74-75, 80-82, 84-86 and 88 have been canceled. Claims 69, 70, 83 and 87 have been withdrawn from consideration. No claims have been allowed.

STATUS OF AMENDMENTS

Claims 39-52, 61-68, 71-73 and 76-79 were finally rejected in the Office Action of July 18, 2003. An Amendment After Final Rejection was filed on January 20, 2004. An advisory action was mailed to appellants on March, 12, 2004, and the advisory action stated that the Amendment After Final Rejection would be entered for purposes of this appeal. The Amendment

After Final Rejection did not result in the amendment of any claims, but did amend the specification.

SUMMARY OF INVENTION

Atherosclerosis, a principal cause of heart attacks among adults in the United States, results from the restricted flow of arterial blood due to the accumulation of fibrous plaque over time in the arterial lumen. Death or incapacitation of the subject may result from the rupture of the fibrous cap of the plaque, causing hemorrhage, thrombosis and occlusion of the artery. The fibrous plaque is formed from fatty streaks which develop into lesions composed predominantly of layers of smooth muscle cells, lipid-filled macrophages and T cells. The earliest stages of atherosclerosis occur when migrating monocytes and T lymphocytes bind to the lumen of the arterial wall. Atherosclerosis is a chronic, long term condition, as distinguished from more acute conditions such as local inflammation. Page 1, line 17 to page 2, line 22. Appellants have found that P-selectin is implicated in the origins of atherosclerosis as a result of the mediation of platelet or endothelial cell binding and adhesion to monocytes. Page 5.

The claimed invention is directed to methods for treating or inhibiting atherosclerosis in a mammal by administering an effective amount of an antibody to the mammal. The antibody is capable of inhibiting an interaction between P-selectin and a ligand of P-selectin, and between E-selectin and a ligand of E-selectin. Page 9, lines 10-34.

ISSUES

The issues to be decided in this appeal are as follows:

1. Whether claims 39, 42-52, 61-65, 68, 74 and 76-79 are unpatentable under 35 U.S.C. 102(b) as anticipated by Furie et al. (EP 0496832).

2. Whether claims 39-52, 61-68, 71-74 and 76 are unpatentable under 35 U.S.C. 102(b) as anticipated by Palabrica et al. (WO 93/06863).

3. Whether claims 39-52, 61-65, 68 and 73-74 are unpatentable under 35 U.S.C. 102(a)(e) as anticipated by McEver et al (U.S. Patent No. 5,378,464).

4. Whether claims 39-52, 61-68, 71-74 and 76-79 are unpatentable under 35 U.S.C. 103(a) as obvious over Furie et al. and/or Palabrica et al. and/or McEver et al., in view of the known use of combination therapies in the treatment of atherosclerosis as taught by Coller et al. (U.S. Patent No. 5,976,532), further in view of the known art underlying lesions of atherosclerosis and known treatments of atherosclerosis (pages 1-2 of the specification), and further in view of the known modes of administration practiced by the ordinary artisan (pages 12-16 of the specification).

GROUPING OF CLAIMS

Claim 39 is the sole independent claim in the application on appeal, and the remaining claims are dependent on claim 39. Accordingly, all claims on appeal can be grouped together, and all claims stand or fall together.

ARGUMENT

I. Rejection of Claims 39, 42-52, 61-65, 68, 74 and 76-79 over Furie et al.

Claims 39, 42-52, 61-65, 68, 74 and 76-79 have been rejected under 35 U.S.C. 102(b) as anticipated by Furie et al. (EP 0496832). Appellants respectfully request reversal of this rejection by the Board.

Furie et al. relates to a method for inhibiting the binding of P-selectin bearing cells, such as platelets, and a P-selectin ligand through the use of an antibody to P-selectin ((PADGEM).

The Furie et al. reference does not disclose, however, that P-selectin antibodies can be used to inhibit the interaction of **both** P-selectin and a ligand of P-selectin, and E-selectin and a ligand of E-selectin, as required by all of the present claims. In fact, Furie et al is completely silent as to E-selectin (ELAM-1) and E-selectin ligand binding, and accordingly, the reference fails to enable antibodies which are capable of inhibiting the interaction of E-selectin and a ligand of E-selectin..

The Examiner states that the antibodies of Furie et al. inherently inhibit the interaction of E-selectin and a ligand of E-selectin as presently claimed by applicants. The Examiner further states that this is a "mechanism of action" that lacks patentable significance, and that the recognition of "latent properties" does not result in patentability. Appellants strongly disagree with these propositions as applied to the claims on appeal.

It is appellants' position that the mere fact that an antibody inhibits P-selectin, as in Furie et al., does not inherently mean that it will also inhibit E-selectin as contended by the Examiner. The present claims specifically recite that the antibodies of the invention must have the property of being capable of inhibiting **both** P-selectin (PADGEM) and E-selectin binding. This is neither a latent property nor an inherent characteristic of all anti-P-selectin antibodies, as explained in more detail below. In fact, this is a functional characteristic of appellants' claimed antibodies that makes the present antibodies unique and patentable in view of the antibodies disclosed in Furie et al.

In support of the above proposition, enclosed in Exhibit A are the pertinent portions of the Declaration Under 37 C.F.R. 1.132 of Denisa Wagner, a co-inventor of the above-identified patent application. Dr. Wagner's credentials are summarized in the exhibits section of the Declaration. The Declaration was filed in parent application USSN 08/948,393, filed October 10, 1997, in response to a similar rejection made in that application. The pertinent portions of the Declaration, for purposes of the issues to be decided in this appeal, are paragraphs 1-5 and 13-15. In summary, these paragraphs state that (1) an agent that inhibits P-selectin binding does not also inherently inhibit E-selectin, and (2) it is unexpected than an agent that inhibits both P-selectin and E-selectin binding would have enhanced effectiveness in treating atherosclerosis.

The Wagner Declaration is referred to in this appeal submitted by appellants to rebut the Examiner's contention that the inhibition of both P-selectin and E-selectin is inherent in the antibodies described in Furie et al. In view of the Declaration, appellants submit that there is no basis for drawing any such conclusion. Appellants' note that the Wagner Declaration is not contradicted by any of the statements made in the references cited in this appeal.

II. Rejection of Claims 39-52, 61-68, 71-74 and 76 over Palabrica et al.

Claims 39, 42-52, 61-65, 71-74 and 76 have been rejected under 35 U.S.C. 102(b) as being anticipated by Palabrica et al. (WO 93/06863). Appellants request the Board to reverse this rejection.

Palabrica et al. relates to the use of P-selectin antibodies to inhibit vascular narrowing associated with post-angioplasty restenosis following a surgical procedure. There is no disclosure in the Palabrica et al. reference regarding the use of P-selectin antibodies to prevent or inhibit atherosclerosis. Moreover, there is no disclosure in the Palabrica et al. reference that the antibodies disclosed therein must be capable of inhibiting both P-selectin and E-selectin binding to their respective ligands.

The Examiner has asserted that appellants' claimed methods would be inherent from the methods described in Palabrica et al. which involve the inhibition of vascular narrowing following angioplasty as described in that reference. Appellants' point out that the procedures described in the Palabrica et al. reference are directed to the treatment of restenosis rather than the treatment of atherosclerosis. Atherosclerosis is a long term chronic condition characterized by the build up of fibrous plaque in the arterial walls of a subject over an extended period of time. Restenosis, on the other hand, is short term or acute condition resulting from a surgical procedure performed on a patient. Moreover, there is no basis for assuming that the antibodies of Palabrica et al. would be effective for inhibiting both PADGEM and E-selectin binding since, as discussed above, the Palabrica et al. reference only describes activity toward PADGEM.

III. Rejection of Claims 39-52, 61-65, 68 and 73-74 over McEver at al.

Claims 39-52, 61-65, 68 and 73-74 have been rejected under 35 U.S.C. 102(a)(e) as being anticipated by McEver et al. Appellants respectfully request the Board to reverse this rejection.

McEver et al. describes a method for modulating an inflammatory response in a patient by treating the patient with inhibitors for GMP-140, such as through the use of GMP-140 antibodies. The inflammatory responses described in the reference include circulatory shock, organ transplant rejection, myocardial infarction and acute respiratory distress syndrome. Atherosclerosis is not an inflammatory condition as that term is used in the McEver et al. reference.

In cols. 20 and 21, the McEver et al. reference contains a list of various inflammatory responses that could conceivably be treated with antibodies to GMP-140. Although atherosclerosis is included in that list, the list also includes such diverse conditions as sepsis, adult respiratory syndrome, tumor metastasis, and intravascular coagulation. This listing of diseases is strictly conjectural on the part of the patentee, and one skilled in the art would certainly not consider this list as enabling for any of the listed diseases. For instance, the patentee states that "platelet-leukocyte interactions are **believed to be** important in atherosclerosis (emphasis supplied)". See col. 21, lines 53-54. In any event, it is clear that the patentee intends to treat the inflammatory effects of these various diseases, rather than the underlying conditions themselves. See, for instance, col. 21, lines 62-64, where the patentee states that the use of the glycoprotein antibody is for the purpose of controlling an inflammatory response.

Further, there is no indication from the McEver et al. reference that GMP-140 antibodies would also be useful to prevent the binding of E-selectin to a ligand of E-selectin as required in the present claims. McEver et al. draw a distinction between GMP-140 and ELAM-1 as shown in col. 17 of the reference.

IV. Rejection of Claims 39-52, 61-68, 71-74 over Furie et al. and/or Palabrica et al. and/or McEver et al., in view of Coller et al. and the present specification.

Claims 39-52, 57, 61-68, 71-74 and 76-79 stand rejected under 35 U.S.C. 103(a) as obvious over the combination of Furie et al., and/or Palabrica et al., and/or McEver et al., in view of known combination therapies used in the treatment of atherosclerosis as taught by Coller et al. (U.S. Patent No. 5,976,532), and/or admissions contained in appellants' specification. Appellants request the Board to reverse this rejection.

The Furie et al., Palabrica et al. and McEever et al. references have been discussed above, and have been distinguished from the present invention for reasons presented therein. These references, either singly or in combination, fail to teach or suggest the methods and therapeutic agents of the present invention since the references do not disclose P-selectin antibodies which are effective for inhibiting both P-selectin and E-selectin binding with their respective ligands. These deficiencies are not cured by the Coller et al. reference which has been cited only for its disclosure of the use of combination therapies and vessel corrective techniques.

Appellants further assert that the present specification does not contain an admission that any aspect of the presently claimed invention is known in the art. The statement on pages 12-16 of the specification regarding administration of the therapeutic agent is part of the description of the methods for using the invention, and is not an acknowledgement that the present invention has been used in this manner by others.

CONCLUSION

Claims 39-52, 61-68, 71-73 and 76-79 are deemed to be patentable over the Furie et al., Palabrica et al., McEver et al. and Coller et al. references, and to overcome the remaining grounds of rejection in this application. All of the cited references, properly construed, relate to the treatment of short term acute conditions, which may also result form the immediate effects of longer term diseases, such as atherosclerosis. In any event, the treatment of the longer term chronic condition, which is the hallmark of atherosclerosis, is not within the contemplation or scope of these references. Appellants submit that a fair and objective reading of these references would support this position.

Accordingly, for the reasons presented in this brief, appellants respectfully urge the Board to reverse the rejections made in the Final Office Action, and to allow all of the appended claims.

Appellants hereby authorize the Commissioner, to debit the $330.00 fee for filing this appeal brief from Appellant's Deposit Account No. 18-1945. If there are any other fees not accounted for above, Appellants hereby authorize the Commissioner to charge the fee to Deposit Account 18-1945.

Respectfully submitted,

ROPES & GRAY

Date: 5/14/04

William Gosz

William G. Gosz
Reg. No. 27,787
Attorney for Appellants
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One International Place
Boston, MA 02110

APPENDIX

39. A method for treating or inhibiting atherosclerosis in a mammal comprising:

providing a P-selectin antibody for inhibiting an interaction between P-selectin and a ligand of P-selectin, and between E-selectin and a ligand of E-selectin; and

administering said antibody to a mammal in need of such treatment so as to cause such inhibition to occur.

40. The method of claim 39 wherein said antibody is administered to the mammal in conjunction with a vessel-corrective technique.

41. The method of claim 40 wherein the vessel corrective technique is selected from the group consisting of angioplasty, a stenting procedure, atherectomy, and bypass surgery.

42. The method of claim 39 wherein said P-selectin is on a cell.

43. The method of claim 39 wherein said cell is an endothelial cell.

44. The method of claim 42 wherein said cell is a platelet.

45. The method of claim 39 wherein said E-selectin is on an endothelial cell.

46. The method of claim 39 wherein said ligand of P-selectin comprises a carbohydrate.

47. The method of claim 39 wherein said ligand of P-selectin comprises a glycoprotein.

48. The method of claim 39 wherein said ligand of P-selectin is selected from the group consisting of sialyl-Lewis x, sialyl-Lewis a, sialyl-Lewis x-pentasaccharide,

polyactosaminoglycan, carbohydrate containing 2,6 sialic acid, Lewis x 3'-0-sulfate, heparin oligosaccharides, PSGL-1, 160 kD monospecific P-selectin ligand and lysosomal membrane glycoproteins.

49. The method of claim 39 wherein said ligand of P-selectin is on a cell selected from the group consisting of monocytes, neutrophils, eosinophils, CD4+ T cells, CD8+ T cells, and natural killer cells.

50. The method of claim 39 wherein said ligand of P-selectin is on a leukocyte.

51. The method of claim 50 wherein said leukocyte is a neutrophil.

52. The method of claim 50 wherein said leukocyte is a monocyte.

61. The method of claim 39 wherein said antibody is an inhibitor of a molecule required for the synthesis, post-translational modification or functioning of said P-selectin or said ligand of P-selectin.

62. The method of claim 39 wherein said antibody inhibits interaction between said P-selectin and said ligand of P-selectin or between E-selectin and said ligand of E-selectin so as to inhibit formation of an atherosclerotic streak, or reverse a formed atherosclerotic fatty streak.

63. The method of claim 39 wherein said antibody inhibits interaction between said P-selectin and said ligand of P-selectin or between E-selectin and said ligand of E-selectin so as to inhibit formation of an atherosclerotic intermediate lesion, or reverse a formed atherosclerotic intermediate lesion.

64. The method of claim 39 wherein said antibody inhibits interaction between said P-selectin and said ligand of P-selectin or between said E-selectin and said ligand of E-selectin so as to

inhibit formation of an atherosclerotic fibrous plaque, or reverse a formed atherosclerotic fibrous plaque.

65. The method of claim 39 wherein said antibody inhibits interaction between said P-selectin and said ligand of P-selectin or between said E-selectin and said ligand of E-selectin so as to inhibit formation of an atherosclerotic lesion after a surgical procedure for inhibiting restenosis.

66. The method of claim 39 wherein said administering occurs prior to formation of an atherosclerotic lesion.

67. The method of claim 39 wherein said administering occurs subsequent to formation of an atheroschlerotic lesion.

68. The method of claim 39 wherein said mammal is a human.

71. The method of claim 39, wherein said antibody is administered at a dose of about 0.01 to about 200 mg/kg body weight.

72. The method of claim 39, wherein said antibody is administered at a dose of about 1 to about 100 mg/kg body weight.

73. The method of claim 39, wherein said antibody further inhibits interaction between L-selectin and a ligand of L-selectin.

76. The method of claim 39, wherein said antibody is administered in sequential exposures over a period of hours, days, weeks, months or years.

77. The method of claim 39, wherein said antibody is administered repeatedly, or by controlled release delivery system.

78. The method of claim 39, wherein the antibody is administered in combination with other therapeutic agents.

79. The method of claim 39, wherein the antibody is administered as a pill, as an injection or as an implant.

Express Mail No. EH966441983 ATTORNEY DOCKET NO. 23134-24

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Denisa D. Wagner and Robert C. Johnson
Serial No. Not yet assigned
Filed: October 10, 1997
For: METHOD FOR TREATING AND PREVENTING ATHEROSCLEROSIS

BOX FWC
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

DECLARATION UNDER 37 C.F.R. §1.132

I, Denisa D. Wagner, declare that:

1. I am a Professor in the Department of Pathology at Harvard Medical School, and Senior Investigator at The Center for Blood Research, Boston, Massachusetts. My Curriculum Vitae is attached as Appendix I. I am an inventor of the above-identified application. I am highly skilled in the art regarding the subject matter to which the above-identified application pertains and am aware of the knowledge of the ordinary person skilled in the art.

2. I have examined the Office Action for the parent application Serial No. 08/377,798 dated November 13, 1996, for the above-identified continuation application. I am familiar with the present claims of this application which are directed to methods for treating or inhibiting atherosclerosis in a mammal by administering an agent which inhibits interaction between P-selectin and a ligand P-selectin and between E-selectin and a ligand of E-selectin.

3. I am familiar with the prior art cited by the Examiner: Kogan et al., Rao et al., Seekamp et al., Ross, Rohrer et al. and DeAmbrosi.

4. Based on my background, training and experience in this field, it is my opinion that it is not inherent that every agent which inhibits interaction between P-selectin and a P-selectin ligand also inhibits interaction between E-selectin and an E-selectin ligand. It was known in the art at the time that the parent application was filed, that in fact there are agents which inhibit P-selectin but not E-selectin, and agents which inhibit E-selectin but not P-selectin. See, e.g., attached Exhibit A: Cecconi et al., J. Biol. Chem. 269:15060-15066 (1994)(describes agent which inhibits P-selectin but not E-selectin), and Exhibit B: Lenter et al., J. Cell Biol. 125:471-481 (1994)(describes ligands which bind to P-selectin but not to E-selectin and other ligands which bind to E-selectin but not to P-selectin).

5. Based on my background, training and experience in this field, it is my opinion that it was not obvious at the time that the parent application was filed for one skilled in the art to select agents which inhibit both P-selectin and E-selectin for enhanced effectiveness in treating atherosclerosis. It was not known in the art at that time that both E- and P-selectin are involved in cardiovascular disease and chronic inflammation.

6. Based on my background, training and experience in this field, it is my opinion that an important distinction exists between "acute inflammation" and "chronic inflammation." See, e.g., attached Exhibit C: Robbins, in "Pathologic Basis of Disease," 5th Ed., R.S. Cotran, M.D., V. Kumar, M.D. and S.L. Robbins, M.D., W.B. Saunders Co., Philadelphia, PA, pp. 51-76 (1994). "Acute inflammation" is of relatively short duration and is involved in processes such as wound repair, infection and reperfusion injury. It involves mainly recruitment of neutrophils. "Chronic inflammation," on the other hand, is of longer duration and is associated predominantly with the recruitment of monocytes and T-cells. As is known to one skilled in the art, atherosclerosis is a special example of chronic

inflammation. This specificity of recruitment, combined with smooth muscle cell proliferation and dependence on cholesterol ingestion by the monocytes/macrophages, makes atherosclerosis a unique process. At the time that the parent application was filed, there were no known adhesion receptors that were specific for recruitment of monocytes and T-cells.

7. Based on my background, training and experience in this field, it is my opinion that it was known to one skilled in the art at the time that the parent application was filed, that P-selectin was a receptor that mediated rolling of many types of white blood cells, was rapidly expressed on activated cells, and was stored in preformed granules that could be rapidly released from these cells. Moreover, as was known by one skilled in the art at the time, P-selectin was involved in early recruitment of neutrophils in experimentally-induced inflammation. See attached Exhibit D: Mayadas et al., Cell 74:541-554 (1993)(recruitment of neutrophils was delayed in P-selectin-deficient mice for two hours and then occurred at a rate identical to wild-type mice). Similarly, delay in the recruitment of neutrophils in wound healing has been reported to occur only in the first two hours in P-selectin-deficient mice after injury. See attached Exhibit E: Subramaniam et al., Am. J. Pathology 150:1701-1709 (1997). And, recruitment of macrophages three to seven days post wounding has been reported to be normal in P-selectin-deficient mice, with wound healing occurring at the same rate as in wild-type mice. See attached Exhibit E: Subramaniam et al., Am. J. Pathology 150:1701-1709 (1997). It is my opinion that to a person skilled in the art, these results indicate that P-selectin plays a role in acute inflammation and injury, but not in chronic processes such as atherosclerosis.

8. Based on my background, training and experience in this field, to the best of my knowledge the first published indication that P-selectin plays a role in long-term chronic inflammation

came in 1995, after the filing date of the parent application. See attached Exhibit J: Johnson et al., Blood 86:1106-1114 (1995) which showed reduced macrophage recruitment 48 hours after induction of experimental inflammation. See also attached Exhibit F: Subramaniam et al., J. Exp. Med. 181:2277-2282 (1995). This paper reported that recruitment of inflammatory cells, including $CD4^+$ T cells, in a contact hypersensitivity response, was reduced in P-selectin-deficient mice. This result was a big surprise to those skilled in the art.

9. Based on my background, training and experience in this field, to the best of my knowledge it was not until 1997, after the filing date of the parent application, that the first published report appeared demonstrating a role for any adhesion receptor molecule, and specifically for P-selectin, in atherosclerosis. See attached Exhibit G: Johnson et al., J. Clin. Invest. 99:1037-1043 (1997).

10. Based on my background, training and experience in this field, it is my opinion that a role for P-selectin in chronic inflammation such as atherosclerosis was contrary to the state of knowledge of those skilled in the art at the time that the parent application was filed, and was certainly not "obvious" to those skilled in the art.

11. Based on my background, training and experience in this field, it is my opinion that a role for E-selectin in chronic inflammation such as atherosclerosis was not experimentally supported at the time that the parent application was filed. Indeed, even as of the instant date, no defects in any inflammatory or wound healing models have been reported for E-selectin-deficient mice unless antibodies inhibitory of P-selectin are also used. See attached Exhibit K: Labow et al., Immunity 1:700-720 (1994)(published after the filing date of the parent application).

12. Based on my background, training and experience in this field, to the best of my knowledge, it was not until 1996, after the filing of the parent application, that it was reported, by myself and others, that major defects existed in P- and E-selectin double deficient mice. See attached Exhibit H: Frenette et al., Cell 84:563-574 (1996), and attached Exhibit I: Bullard et al., J. Exp. Med. 183:2329-2336 (1996). These papers showed conclusively for the first time that the two endothelial selectins, P and E together, are crucial for leukocyte recruitment to sites of inflammation. Prior to these papers, these selectins were known to be involved in leukocyte rolling (with minor or no consequences on leukocyte recruitment)(see attached Exhibit D: Mayadas et al., Cell 74:541-554 (1993)), and it is my opinion that it was believed by persons skilled in the art that it was the adhesion molecules responsible for leukocyte firm adhesion to endothelium (belonging to the immunoglobulin and integrin family of receptors), that were crucial for the final transmigration of leukocytes to sites of inflammation in the tissues.

13. Based on my background, training and experience in this field, it is my opinion that a role for E-selectin in atherosclerosis had not been shown at the time that the parent application was filed.

14. Attached hereto as Exhibit L are five figures illustrating the results of experiments performed in my laboratory which support the surprising and unexpected results obtained from mice being deficient in both P-selectin and E-selectin, as opposed to being deficient just for P-selectin, in inhibiting atherosclerotic lesions on arterial walls. Experimental protocols were performed as described in Exhibit G: Johnson et al., J. Clin. Invest. 99:1037-1043 (1997). Fig. 1 illustrates that the size of aortic sinus lesions in LDL-receptor (LDLR)-deficient mice on an atherogenic (high cholesterol and

fat) diet is significantly smaller in P- and E-selectin double deficient mice than in wild-type or just P-selectin-deficient mice. Fig. 2 consists of photographs of entire aortae of LDLR-deficient mice on an atherogenic diet, and illustrates that the percentage area of the aortae that have atherosclerotic lesions is significantly smaller in P- and E-selectin double deficient mice than in wild-type mice. Fig. 3 illustrates that there are significantly smaller aortic sinus lesions in LDLR-deficient mice on an atherogenic diet in P- and E-selectin double deficient mice than in wild-type or just P-selecin-deficient mice. Fig. 4 illustrates that the size of atherosclerotic lesions in the aortic sinus of LDLR-deficient mice, as a function of the length of time on an atherogenic diet, is significantly smaller for up to at least 37 weeks, in P- and E-selectin double deficient mice than in wild- type or just P-selectin-deficient mice. Fig. 5 illustrates that the percentage of mice with calcification in the aortic sinus of LDLR-deficient mice on an atherogenic diet, is significantly less in P- and E-selectin double deficient mice than in wild type mice.

15. Based on my knowledge, training and experience in this field, it is my opinion that no combination of the cited prior art teaches or suggests a method for treating or inhibiting atherosclerosis by providing an agent for inhibiting interaction between P-selectin and a ligand of P-selectin and between E-selectin and a ligand of E-selectin. Nor does any combination of the cited prior art suggest the advantages that are present in applicants' invention.

I further declare that statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or

imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Date: 10/8/97

Denisa D. Wagner

DENISA D. WAGNER, Ph.D.

392638_1.WP6

Appendix I

CURRICULUM VITAE

DENISA D. WAGNER, Ph.D.

ADDRESS: The Center for Blood Research
Harvard Medical School
800 Huntington Avenue
Boston, MA 02115
Phone: (617) 278-3344
FAX: (617) 278-3368

BORN: Prague, Czechoslovakia - December 30, 1950; U.S. Citizen

EDUCATION: Universite de Geneve, Switzerland - Biochemistry
Diploma of Biochemistry, 1975, with distinction

Massachusetts Institute of Technology, Cambridge, MA
Biology - Ph.D., 1980

FACULTY POSITIONS:

Professor of Pathology, Harvard Medical School, Boston, MA.
1997-present.

Associate Professor of Pathology, Harvard Medical School, Boston, MA.
1994-1997.

Senior Investigator, The Center for Blood Research, Boston, MA.
1994-present.

Associate Professor of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, MA. 1989-1994.

Associate Professor of Medicine, Tufts University School of Medicine and Member, Special and Scientific Staff, New England Medical Center, Boston, MA. 1987-1994.

Assistant Professor of Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, New York. 1985-1987.

Assistant Professor of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York. 1982-1987.

Senior Instructor in Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York. 1981-1982.

AWARDS: Established Investigator Award, American Heart Association, Biosynthesis of von Willebrand protein by endothelial cells. 1986-1991.

XIth ISTH Congress award in recognition of an outstanding communication, 1987.

MEMBERSHIPS, OFFICES, AND COMMITTEE ASSIGNMENTS IN PROFESSIONAL SOCIETIES:

1980-Present	American Society for Cell Biology
1982-Present	American Society of Hematology
1982-Present	International Society of Thrombosis and Haemostasis
1983-Present	Council on Thrombosis, American Heart Association
1985-Present	International Society of Thrombosis and Haemostasis, subcommittee on von Willebrand factor
1991-1996	American Heart Association, Vascular Wall Biology Study Committee
1992-Present	American Heart Association, Council on Thrombosis Executive Committee
1993-1995	American Heart Association, Council on Thrombosis Long-Range Planning Committee
1994-1996	American Heart Association, Council on Thrombosis Membership Committee (Chairman)
1994-Present	American Association for the Advancement of Science
1994-Present	North American Vascular Biology Organization
1995-1998	American Society of Hematology, Scientific Subcommittee on Thrombosis
1997-Present	North American Vascular Biology Organization, Councilor

EDITORIAL BOARDS:

1993-Present	Molecular Biology of the Cell
1994-Present	Journal of Clinical Investigation

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DENISA D. WAGNER, Ph.D.

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EXHIBIT A

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Inositol Polyanions

NONCARBOHYDRATE INHIBITORS OF L- AND P-SELECTIN THAT BLOCK INFLAMMATION*

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Selectins are cell adhesion molecules known to support the initial attachment of leukocytes to inflamed vascular endothelium through their recognition of carbohydrate ligands such as the tetrasaccharide sialyl Lewis^x (Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAc-). In the present study, we describe the inhibition of L- and P-selectin function by inositol polyanions, simple 6-carbon ring structures that have multiple ester-linked phosphate or sulfate groups. In a purified component competition assay, binding of L- and P-selectin-Ig fusion proteins to immobilized bovine serum albumin-sialyl Lewis^x neoglycoprotein was inhibited by inositol hexakisphosphate ($InsP_6$, IC_{50} = 2.1 ± 1.4 μM and 160 ± 40 μM), by inositol pentakisphosphate ($InsP_5$, IC_{50} = 1.4 ± 0.2 and 260 ± 40 μM), and by inositol hexakissulfate ($InsS_6$, IC_{50} = 210 ± 80 μM and 2.8 ± 0.9 mM); E-selectin-Ig binding was unaffected. Inositol polyanions diminished the adhesion of LS180 colon carcinoma cells to plates coated with L- and P-selectin-Ig but not with E-selectin-Ig. Inositol polyanions blocked polymorphonuclear leukocyte (PMN) adhesion to COS cells expressing recombinant transmembrane P-selectin but not to those expressing E-selectin. In addition, inositol polyanions diminished PMN adhesion to activated endothelial cells under rotation-induced shear stress, a process known to require L-selectin function. *In vivo*, the effects of inositol polyanions were studied in two murine models of acute inflammation. Intravenously administered $InsP_6$ (two doses of 40 μmol/kg) inhibited PMN accumulation in thioglycolate-induced inflammation (55 ± 10% inhibition) and in zymosan-induced inflammation (61 ± 4% inhibition). $InsP_5$ and $InsS_6$ also inhibited inflammation in these models, although higher doses were required for $InsS_6$. In conclusion, inositol polyanions are noncarbohydrate small molecules that inhibit L- and P-selectin function *in vitro* and inflammation *in vivo*.

Selectins are transmembrane glycoproteins containing an N-terminal lectin domain, an epidermal growth factor repeat, and a discrete number of complement regulatory-like repeats (reviewed in Ref. 1). E-selectin is synthesized and expressed by cytokine- and endotoxin-activated endothelial cells with peak expression occurring at 4 h. P-selectin is stored in granules of platelets and endothelial cells and can be rapidly redistributed to the cell surface following activation by certain mediators, including thrombin. The third family member, L-selectin, is constitutively expressed by neutrophils, monocytes, and most lymphocytes and participates in the regulation of leukocyte rolling and lymphocyte homing; a substantial portion of cell surface-expressed L-selectin is shed upon cellular activation. E-, P-, and L-selectin bind sLe^{x1} and related oligosaccharides (1); however, recent studies suggest differences in binding specificities and affinities (2–6). Other studies have highlighted the importance of specific proteins in the presentation of carbohydrate ligands (7–13).

Soluble oligosaccharides related to sLe^x and sLe^a (Neu5Acα2–3Galβ1–3(Fucα1–4)GlcNAc-) can inhibit adhesive functions of selectins (6, 14, 15) and have anti-inflammatory activity in rodent models of P- and E-selectin-dependent inflammation (16–18). Three observations prompted us to look for other classes of selectin inhibitors. First, sLe^x and sLe^a appear to interact relatively weakly with L- and P-selectin. For example, sLe^x concentrations in excess of 5 mM are required to block 50% of L-selectin-dependent interactions *in vitro* (2, 19). Second, the complex structures of these oligosaccharides pose significant obstacles for large-scale synthesis. Third, previous studies have shown that a variety of phosphate- or sulfate-containing carbohydrates can interact with L- and P-selectin (6, 20–29). For example, PPME, the polyphosphomonoester core of *Hansenula holstii O*-phosphonomannan (30), is a blocker of L-selectin-dependent adhesion (20, 25, 26). High concentrations (5–10 mM) of monophosphated monosaccharides can also block L- and P-selectin-dependent adhesion *in vitro* (20, 23, 26, 27). In addition, two highly sulfated macromolecules, fucoidan, a polysaccharide produced by brown algae, and heparin, a glycosaminoglycan produced by mast cells, interact with both L- and P-selectin *in vitro* (6, 21, 22, 28, 29) and

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[1] The abbreviations used are: sLe^x, sialyl Lewis x; sLe^a, sialyl Lewis a; $InsP_6$, D-*myo*-inositol 1,2,3,4,5,6-hexakisphosphate; $InsS_6$, D-*myo*-inositol 1,2,3,4,5,6-hexakissulfate; $InsP_5$, D-*myo*-inositol 1,3,4,5,6-pentakisphosphate; Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; Ins(3,5,6)P_3, D-*myo*-inositol 3,5,6-trisphosphate (equivalent to L-Ins (1,4,5)P_3); GroPIns(4,5)P_2, 1-(α-glycerophospho)-D-*myo*-inositol 4,5-bisphosphate; GroPIns, 1-(α-glycerophospho)-D-*myo*-inositol; $InsP_1$, D-*myo*-inositol 1-monophosphate; Glc(2,3,4,6)P_4, D-glucose 2,3,4,6-tetrakisphosphate; mAb, monoclonal antibody; DPBS, Dulbecco's phosphate-buffered saline (containing Ca^{2+} and Mg^{2+}); BSA, bovine serum albumin; BSA-sLe^x, bovine serum albumin conjugated to sLe^x; PMN, polymorphonuclear leukocytes; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells; HSA, human serum albumin; BAL, bronchoalveolar lavage; PBS, phosphate-buffered saline.

inhibit leukocyte rolling (31–33) and inflammation *in vivo* (29). Together, these observations suggested the possibility that multiple phosphate or sulfate groups can contribute to the formation of ligands for at least two of the selectins. In the present study, we attempt to better define selectin interactions with polyphosphated or polysulfated structures. We focused on inositol polyanions, simple 6-carbon ring structures derived from D-*myo*-inositol (1,2,3,5-trans-4,6 cyclohexanehexol) by esterification with phosphate or sulfate groups (Fig. 1). The effects of inositol polyanions on selectin function were examined using a direct binding assay (competition ELISA), as well as several assays measuring selectin-dependent cell adhesion. Finally, inositol polyanions were tested *in vivo* in two mouse models of peritoneal inflammation and in a rat model of lung inflammation.

MATERIALS AND METHODS

Chemicals—$InsP_6$ (dodecasodium salt), $InsS_6$ (hexapotassium salt), $Ins(1,4,5)P_3$ (potassium salt), inositol 1-monophosphate (cyclohexylammonium salt), *myo*-inositol, 2,3-bisphosphoglycerate (pentasodium salt), pentasodium tripolyphosphate hexahydrate ($Na_5P_3O_{10}$), and trisodium trimetaphosphate ($Na_3P_3O_9$) were from Sigma. $InsP_6$ was also purchased from Calbiochem (La Jolla, CA), as were $InsP_5$ (decasodium salt), $GroPIns(4,5)P_2$ (trilithium salt), $Ins(3,5,6)P_3$ (trilithium salt), and GroPIns (lithium salt). D-glucose 2,3,4,6-tetrakisphosphate was synthesized starting from α-D-glucose, which was converted to the benzyl α-D-glucopyranoside. Polyphosphitylation with dibenzyl *N,N*-diethyl phosphorous amidite and tetrazole (34) followed by *in situ* oxidation with peracetic acid gave the fully protected benzyl α-D-glucopyranoside 2,3,4,6-tetrakis (dibenzyl) phosphate in good yield (73%). Benzyl protecting groups were quantitatively removed by catalytic hydrogenation with H_2 on Pd/C (10%) to give pure D-glucose 2,3,4,6-tetrakisphosphate as the free acid. All products gave satisfactory ^1H NMR, ^{31}P NMR, and fast atom bombardment mass spectra.

Stock solutions to be used for animal experiments were freshly prepared in sterile pyrogen-free 0.9% NaCl (saline; Abbott Laboratories, North Chicago, IL), adjusted to pH 7.4 with HCl, and sterilized by 0.22-μm filtration. Endotoxin content was tested using a quantitative chromogenic limulus amebocyte lysate assay (LAL, Whittaker Bioproducts, Inc., Walkersville, MD).

Proteins and Antibodies—Selectin-immunoglobulin fusion proteins (selectin-Ig) are recombinant chimeric molecules containing the lectin domain, epidermal growth factor repeat, and one (L-selectin-Ig), two (P-selectin-Ig), or six (E-selectin-Ig) complement regulatory repeats coupled to the hinge, CH2, and CH3 regions of human IgG1 (3, 6, 35–37). Selectin-Ig cDNAs in pCDM7 and pNUT vectors allowed transient expression in COS-1 cells (38) and stable expression in baby hamster kidney cells (39), respectively. Selectin-Igs were affinity-purified from culture media using protein A-agarose (Pierce Chemical Co.) or protein G-Sepharose (Pharmacia LKB Biotechnology Inc.) (38). BSA-sLe^x was kindly provided by Chembiomed, LTD (Edmonton, Alberta, Canada). Horseradish peroxidase-conjugated goat anti-human IgG (Fc-specific) antibody was from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Fluorescein-conjugated anti-human IgG antibody was from Cappel (Durham, NC). BSA (Pentex Fraction V, protease-free) was from Miles, Inc. (Kankakee, IL). Anti-E-selectin mAb H18/7 (protein A-purified antibody) was generated by immunization of mice with human endothelial cells (40). The following murine mAbs were provided as gifts: anti-P-selectin mAbs G1 (protein A-purified antibody, from R. McEver, Oklahoma City, OK) (41) and anti-L-selectin mAb LAM 1.3 (ascites, from T. Tedder, Boston, MA) (42).

Competition ELISA—Competition ELISAs using P- and E-selectin-Ig (10–30 nM) were performed essentially as described (6). Briefly, polystyrene microwell plates (cat. 25801, Corning Glass, Newark, CA) were coated with unconjugated BSA or BSA-sLe^x neoglycoprotein (0.11 μg/well in 75 μl of 50 mM sodium carbonate/bicarbonate buffer, pH 9.5) by incubation overnight at 4 °C. Wells were washed and blocked with 20 mg/ml BSA in assay buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$). P- and E-selectin-Ig and serially diluted test compounds were incubated in BSA-sLe^x-coated wells for 3 h at room temperature. After washing with assay buffer, peroxidase-conjugated goat anti-human IgG antibody (1:3000) was added to the wells and incubated for 30 min. Wells were washed and incubated with a chromogenic substrate for peroxidase (o-phenylene-diamine dihydrochloride, 0.8 mg/ml) in 50 mM sodium citrate, 50 mM sodium phosphate buffer (pH 5.0) containing 0.015% (vol/vol) H_2O_2. Bound selectin-Ig was determined by measuring the optical density at 450 nm at intervals of 12–30 s in a V_{max} microplate reader (Molecular Devices, Inc., Menlo Park, CA); an endpoint determination at 490 nm was made after stopping the color development in the linear range by addition of 4 N H_2SO_4. Specific binding to BSA-sLe^x was determined by subtracting the signal generated in wells coated with unconjugated BSA incubated with 20 nM solutions of selectin-Ig (typically less than 10% of the maximal signal). For L-selectin-Ig (2), 20 nM fusion protein was allowed to form multimeric aggregates with peroxidase-conjugated anti-immunoglobulin antibody (1:6,000) for 30 min before incubation with the inhibitors on the BSA-sLe^x-coated plate. Subsequent steps were as described above. IC_{50} values (concentrations of compound that reduced selectin-Ig binding to 50% of the maximal) were calculated by fitting data from a titration curve to the equation: fraction of maximal binding = $IC_{50} \div (IC_{50}$ + [compound]) using nonlinear least squares analysis software (Origin, Microcal Inc., Northampton, MA).

Cells and Culture Conditions—Human LS180 colon carcinoma cells, human HL60 promyelocytic leukemia cells, and SV40-transformed simian kidney fibroblast COS-1 cells were purchased from American Type Culture Collection (Rockville, MD) and maintained in culture as recommended. Baby hamster kidney cells were cultured in Dulbecco's modified Eagle's medium/F-12 (Whittaker) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and subcultured using versene (Life Technologies, Inc.). Primary cultures of human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics Corp. (San Diego, CA). HUVEC were grown in Medium 199 (Life Technologies, Inc.) containing 20% FBS, 50 μg/ml endothelial cell growth supplement, and 100 μg/ml heparin (Sigma) and subcultured (1:3 split ratio) using trypsin/versene (Life Technologies, Inc.). HUVEC were used for adhesion experiments at passage 2–4. PMN were prepared from EDTA-anticoagulated blood by sodium metrizoate-dextran density gradient centrifugation (Polymorphprep, Nycomed Pharma As, Oslo, Norway) and washed twice with DPBS (without Ca^{2+} and Mg^{2+}, Whittaker) at room temperature. Contaminating red blood cells were lysed by a brief exposure (30 s) to hypotonic buffer (ice-cold DPBS diluted 1:10 in sterile distilled water) followed by the addition of 1/10 of a volume of sterile hypertonic DPBS (10 ×, Whittaker). PMN were washed by centrifugation in ice-cold DPBS (without Ca^{2+} and Mg^{2+}) and suspended in ice-cold 1% human serum albumin (HSA; Cat. 521100, Alpha Therapeutic Corporation, Los Angeles, CA) in RPMI 1640 medium (Whittaker).

LS180 Cell Adhesion to Protein A-captured Selectin-Ig—Cell adhesion assays on immobilized selectin-Ig were performed essentially as described (3, 6). Briefly, Nunclon Terasaki microwell plates (Cat. 136528, Nunc, Naperville, IL) were coated overnight at 4 °C with 5 μl/well 50 mM sodium carbonate buffer (pH 9.5) containing recombinant protein A (10 μg/ml, Chemicon, Temecula, CA). Protein A-coated plates were washed with DPBS, incubated for 1 h with L-, P-, or E-selectin-Ig in DPBS (20, 20, and 5 μg/ml, respectively), washed again, and blocked with DPBS containing 1% HSA. LS180 cells were harvested by brief trypsinization, washed twice by centrifugation, and suspended at 1.5×10^6 cells/ml in DPBS containing 1% HSA; 5 μl/well of the suspension was added to the wells and incubated for 30 min at 4 °C. After washing to remove unbound cells, adherent cells were fixed with glutaraldehyde (2.5% in DPBS) and counted microscopically. In each experiment, wells coated with CD8-Ig fusion protein were used as control. To measure inhibition of cell adhesion, 5 μl/well of serially diluted solutions of inositol or inositol polyanions in DPBS were added to selectin-Ig-coated plates and incubated for 30 min at 4 °C before the addition of LS180 cell suspension.

PMN Adhesion to COS Cells Transfected with cDNAs Encoding L-, P- and E-selectin—pCDM7/pCDM8 vectors containing cDNAs encoding full-length transmembrane forms of L-, P-, or E-selectin were transfected into COS-1 cells using DEAE-dextran as described (38). After 24 h, transfected cells were harvested by brief trypsin treatment, transferred to coverslips coated with 0.1% gelatin (G8–500, Fisher, Pittsburgh, PA), and cultured in 24-well culture plates for 48–72 h to allow for cell surface expression of selectins. Wells were washed with DPBS and incubated with 0.5 ml of DPBS-1% HSA alone or containing inositol or inositol polyanions for 30 min at 4 °C. PMN suspensions (0.5 ml at 2×10^6 cells/ml) were then added and allowed to adhere for 30 min at 4 °C. Nonadherent PMN were removed by immersion of the coverslips in DPBS. Adherent cells were fixed with 2.5% glutaraldehyde in DPBS and counted. PMN adhesion was quantitated as number of rosettes (COS cells with three or more bound PMN) per 100 transfected COS cells. Transfection efficiency (typically 10–25%) was assessed in each experiment by incubating COS cells with mAbs specific for L-, P-, and E-selectin, followed by reaction with a fluorescein-conjugated anti-im-

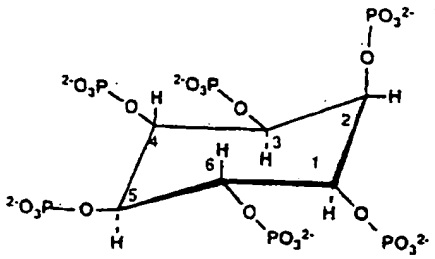

FIG. 1. **Structure of D-*myo*-inositol 1,2,3,4,5,6-hexakisphosphate ($InsP_6$).**

munoglobulin antibody. The same protocol was used for adhesion experiments performed with LS180 and HL60 cell suspensions.

PMN Adhesion to Activated Endothelial Monolayers—HUVEC were grown to confluence within 2.2 cm diameter circles (25) on glass microscope slides coated with 0.1% gelatin. Monolayers were washed with DPBS and incubated for 4–8 h at 37 °C in Medium 199 containing 20% FBS and 200 units/ml human recombinant tumor necrosis factor α (Biogen Research Corp., Cambridge, MA). Monolayers were washed again and incubated for 30 min at 4 °C in Medium 199 containing 5% FBS. After removal of medium, PMN suspension (0.5 ml, 2×10^6/ml) was added and allowed to adhere for 30 min at 4 °C. The adhesion assays were performed both in static and in nonstatic conditions (on a rotating platform, 64 rpm). After washing to remove unbound PMN, glass slides were fixed in DPBS containing 2.5% glutaraldehyde, and adherent PMN were counted microscopically. To determine adhesion-blocking activity, PMN were incubated in RPMI 1640 medium with 5% FBS alone or containing inositol, $InsP_6$, or anti-L-selectin monoclonal antibody LAM 1.3 (25), for 30 min at 4 °C before their addition to activated endothelial monolayers. In some experiments, endothelial monolayers were incubated with anti-E-selectin monoclonal antibody H18/7 (40) for 30 min at 4 °C before the addition of PMN.

Peritoneal Inflammation—Male 4–5-week-old BALB/c mice (20–24 g) were injected intraperitoneally with 1 ml of 3% thioglycollate broth (lot 622462, Clinical Standard Laboratories, Inc., Rancho Dominguez, CA) or with 1 ml of saline containing 0.5 mg of zymosan (Sigma). Control animals were injected with saline. After 40 and 80 min, animals received slow intravenous injections of saline or saline containing $InsP_6$, $InsS_6$, or inositol (0.2 ml/injection). $InsP_6$ was injected at 5–40 μmol/kg/injection (total of 10–80 μmol/kg) and $InsS_6$ and inositol at 40 or 200 μmol/kg/injection (total of 80 or 400 μmol/kg). Mice were sacrificed 120 min after the intraperitoneal injection. Cells within the peritoneal cavity were collected by lavage with 10 ml of ice-cold DPBS containing 10 units/ml of heparin and counted in a hemocytometer. The percentage of PMN was assessed using cytospin preparations (Shandon Inc., Pittsburgh, PA) stained with Wright-Giemsa stain (Diff-Quik, Baxter, McGaw Park, IL). In separate experiments, $InsP_6$ (2–160 μmol/kg) or inositol were given as single 0.2-ml subcutaneous injections 3 min after intraperitoneal injection of thioglycollate. In some experiments, total white blood cell counts were obtained using a Sysmex F-800 hematology analyzer (Baxter), and the percentage of PMN was assessed using smear preparations stained with Diff-Quik. No toxic effects of $InsP_6$ and $InsS_6$ were observed by using the injection protocols described above. Toxicity due to rapid intravenous injection of high dose $InsP_6$ (~600 μmol/kg) has been previously reported (43).

Lung Inflammation—Lung inflammation was induced in rats (male, 200–250 g) by intratracheal injection of endotoxin (5 μg/rat of *Salmonella typhosa* lipopolysaccharide, Sigma, lot 126F4020, in 0.5 ml of saline) as described (44). After 2 h, a first intravenous injection (0.5 ml) of $InsP_6$ (20 μmol/kg) or saline was given; a second injection was given 4 h after the intratracheal injection. Rats were sacrificed 6 h after the intratracheal injection. Bronchoalveolar lavage (BAL) was performed by washing the lungs with 7 ml of PBS injected through a tracheal cannula and repeated 6–7 times/rat. Cells within the BAL fluid were counted with an hematology analyzer, and the percentage of PMN was assessed using cytospin preparations stained with Diff-Quik. Samples of venous blood withdrawn from the tail were used to determine peripheral blood PMN content.

RESULTS

Inositol Polyanions Block the Binding of L- and P-selectin-Ig to BSA-sLe^x in a Competition ELISA—Inositol hexakisphosphate ($InsP_6$, Fig. 1) and inositol hexakissulfate ($InsS_6$), but not inositol, inhibited binding of L- and P-selectin-Ig to immobilized BSA-sLe^x neoglycoprotein (Fig. 2, *A* and *B*). The IC_{50} of $InsP_6$ on L-selectin-Ig binding was 2.1 ± 1.5 μM; $InsS_6$ was less active, with an IC_{50} value of 210 ± 80 μM. $InsP_6$ and $InsS_6$ also blocked P-selectin-Ig binding to immobilized BSA-sLe^x (IC_{50} = 160 ± 40 μM and 2.8 ± 0.9 mM, respectively), whereas they failed to inhibit the binding of E-selectin-Ig at concentrations up to 5 mM (Fig. 2*C*). $InsP_5$ also inhibited L- and P-selectin-Ig binding with IC_{50} values of 1.4 ± 0.2 and 260 ± 40 μM, respectively. As shown in Table I, inositol trisphosphates ($Ins(1,4,5)P_3$, $Ins(3,5,6)P_3$, and $GroPIns(4,5)P_3$) also inhibited L-selectin binding, while $Glc(2,3,4,6)P_4$ was inactive up to 2 mM. Inositol-based structures with low phosphate content (inositol, $InsP_1$, and GroPIns) and polyphosphates (sodium trimetaphosphate, sodium tripolyphosphate, and 2,3-bisphosphoglycerate) did not inhibit selectin binding to BSA-sLe^x up to 2 mM. None of the test compounds inhibited E-selectin-Ig binding to BSA-sLe^x up to 2 mM.

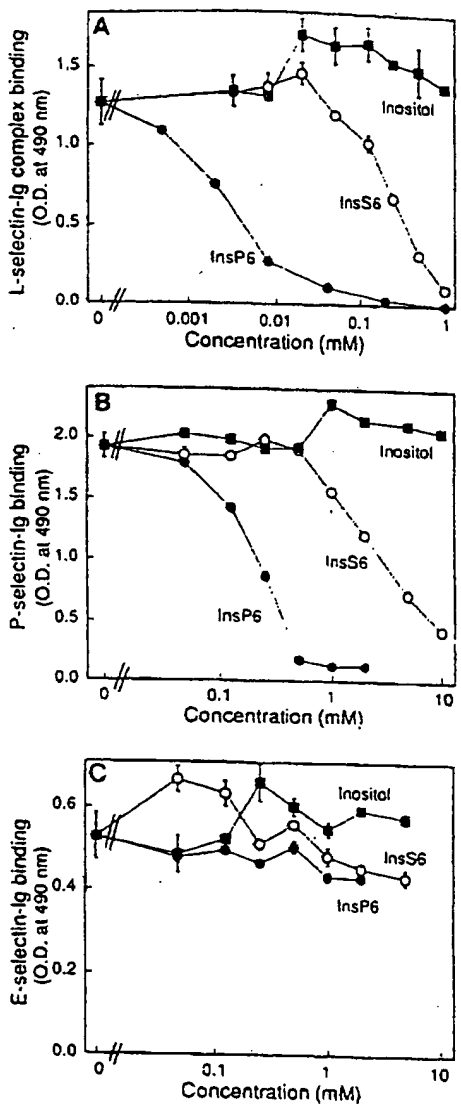

FIG. 2. **Effect of *myo*-inositols on the binding of L- (*a*), P- (*b*), and E-selectin-Ig (*c*) to immobilized BSA-sLe^x in a competition ELISA.** To determine inhibitory activity, $InsP_6$ (●), $InsS_6$ (○), or inositol (■) was added to the selectin-Igs to achieve the indicated concentrations before transfer to microtiter plates. Data shown are the mean and range of optical density measurements made in duplicate wells from a single experiment corrected for background signal, representative of 3–4 separate experiments.

$InsP_6$ and $InsS_6$ Block Cell Adhesion to L- and P-selectin-Ig and to COS Cells Expressing L-and P-selectin—The effect of inositol polyanions on selectin-dependent cell adhesion was as-

TABLE I
Effect of inositol polyanions, inositol, and polyphosphates on L- and P-selectin-Ig binding to BSA-sLe^x

Compound	L-selectin-Ig		P-selectin-Ig	
	IC_{50}[a]	n[b]	IC_{50}	n
	μM		μM	
$InsP_6$	2.1 ± 1.5	4	160 ± 40	4
$Ins(1,3,4,5,6)P_5$	1.4 ± 0.2	3	260 ± 40	3
D-$Ins(3,5,6)P_3$	340 ± 60	3	NC[c]	2
D-$Ins(1,4,5)P_3$	320 ± 110	3	NC	2
$GroPIns(4,5)P_2$	150 ± 40	3	NC	2
GroPIns	NI[d]	2	NI	2
$InsP_1$	NI	2	NI	2
Inositol	NI	4	NI	3
$InsS_6$	210 ± 80	3	2760 ± 890	3
$Glc(2,3,4,6)P_4$	NI	2	NI	2
Tripolyphosphate	NI	3	NI	3
Trimetaphosphate	NI	3	NI	3
2,3-bisphosphoglycerate	NI	2	NI	2

[a] Concentration of compound that resulted in 50% inhibition of L- or P-selectin-Ig binding to BSA-sLe^x as measured in a competition ELISA (see "Materials and Methods"). Data represent the mean ± S.D. of the IC_{50} values obtained in n experiments.
[b] n, number of titration experiments performed for each compound.
[c] NC, not calculated (less than 30% inhibition at 2 mM).
[d] NI, no inhibition (at 2 mM).

sessed using a colon cancer cell line, LS180, that binds to all three selectins.[2] As shown in Fig. 3, $InsP_6$ blocked the adhesion of LS180 cells to plates coated with purified L- and P-selectin-Ig (51 ± 5 and 53 ± 14% inhibition at 60 μM, respectively, 5 experiments) but not with E-selectin-Ig. $InsS_6$ was also effective in blocking LS180 adhesion to L- and P-selectin-Ig but required higher concentrations (43 ± 1 and 45 ± 5% inhibition at 4 mM, respectively, 2 experiments); *myo*-inositol, and 2,3-bisphosphoglycerate were inactive at concentrations up to 5 mM. The effect of inositol polyanions on cell adhesion to full-length transmembrane selectins was examined using COS cells transfected with cDNAs encoding L-, P-, and E-selectin (L-COS, P-COS, and E-COS, respectively). $InsP_6$ displayed a dose-dependent inhibition of LS180 cell adhesion to L-COS and P-COS (blocking at 500 μM was 81 ± 13% and 95 ± 5%, respectively, 2 experiments) but not to E-COS (<5% inhibition). In separate studies, PMN adhered to P-COS and E-COS but showed little binding to L-COS. As depicted in Fig. 4, $InsP_6$ (500 μM) blocked PMN adhesion to P-COS but not to E-COS; similar results were obtained with the promyelocytic cell line HL60 (not shown). Higher concentrations of $InsS_6$ also inhibited PMN adhesion to P-COS (63 ± 7% blocking at 5 mM, 2 experiments) but not to E-COS; inositol had no effect at concentrations up to 10 mM.

$InsP_6$ Inhibits PMN Adhesion to Activated Endothelial Cell Monolayers under Fluid Shear Stress—The effect of inositol polyanions on PMN adhesion to cytokine-activated endothelial monolayers was studied under static and nonstatic conditions. In the absence of fluid shear force (static assay), PMN adhesion to tumor necrosis factor α-activated endothelial cells is inhibited by anti-E-selectin antibodies (40) but not by anti-L-selectin antibodies (25). Under these conditions, $InsP_6$ (500 μM) had little or no effect on PMN adhesion (Fig. 5, *upper panel*). In the presence of rotation-induced shear stress (nonstatic assay), PMN adhesion can be inhibited by both anti-E-selectin and anti-L-selectin antibodies (25). Under these conditions, $InsP_6$ (500 μM) reduced PMN adhesion to tumor necrosis factor α-activated endothelial cells to a similar extent as did anti-L-selectin antibodies (Fig. 5, *lower panel*). Inositol was inactive at concentrations up to 10 mM in both static and nonstatic assays.

[2] G. Mannori, L. Carter, O. Cecconi, K. Hanasaki, C. Corless, A. Aruffo, R. M. Nelson, and M. P. Bevilacqua, manuscript in preparation.

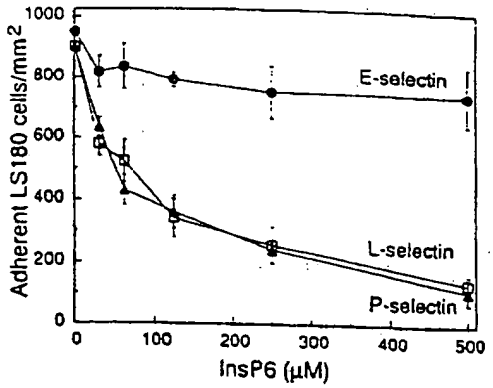

FIG. 3. **Effect of $InsP_6$ on the adhesion of LS180 colon cancer cells to immobilized L- (□), P- (▲), and E-selectin-Ig (●).** $InsP_6$ was added to wells precoated with protein-A-captured selectin-Ig to give the indicated final concentrations. Data presented are mean number of adherent cells/mm^2 ± S.E., counted in quadruplicate wells in a single experiment representative of three. Inositol was inactive up to 10 mM on each of the selectins. $InsS_6$ inhibited L- and P-selectin-dependent cell adhesion at higher concentrations (43 ± 1 and 45 ± 5% inhibition at 4 mM). No LS180 cell adhesion was detected on plates coated with protein A alone or on plates coated with protein A-captured CD8-Ig. LS180 cell adhesion to E-, P-, and L-selectin was inhibited by blocking monoclonal antibodies H18/7, G1, and LAM1.3, respectively.[2]

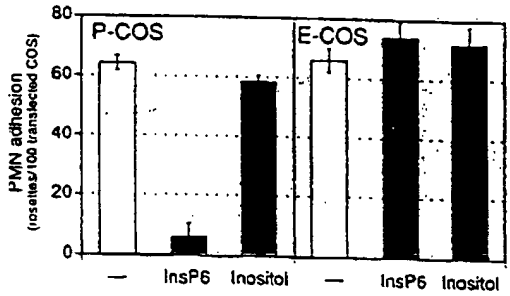

FIG. 4. **Effect of $InsP_6$ or inositol on the adhesion of isolated human PMN to COS cells transfected with P- and E-selectin.** Experiments were conducted as described under "Materials and Methods." Inositol and $InsP_6$ final concentration was 500 μM. Previous experiments showed that the blocking effect was dose-dependent and maximal at concentrations between 250 and 500 μM. Data represent the mean ± S.E. of three separate experiments (five microscopic fields/coverslip with triplicate coverslips were counted in each experiment). Inositol was inactive up to 10 mM. $InsS_6$ inhibited PMN adhesion to P-COS at concentrations higher than 1 mM (63 ± 7% inhibition at 5 mM). Similar results were obtained with HL60 cells.

$InsP_6$ Reduces PMN Accumulation in Experimental Inflammation in Vivo—In mice, peritoneal inflammation induced by injection of thioglycollate results in the accumulation of PMN, the early phase of which is thought to depend largely on L- (45, 46) and P-selectin (47). In this model, $InsP_6$ reduced PMN accumulation in a dose-dependent fashion. Two intravenous injections of 40 μmol/kg resulted in a 55 ± 10% reduction in PMN recovered from the peritoneal cavity at 120 min (Fig. 6*A*). $InsP_5$ (two intravenous injections of 40 μmol/kg) also reduced peritoneal influx of PMN in thioglycollate-stimulated mice (48 ± 6% inhibition, 2 experiments, $p < 0.01$ in both experiments). A single subcutaneous injection of $InsP_6$ (1–160 μmol/kg) acted in a dose-dependent fashion to reduce the number of PMN recovered in the peritoneal cavity of thioglycollate-stimulated animals. The inhibition was maximal between 40 and 160 μmol/kg (45 ± 5% at 40 μmol/kg, 3 experiments). The number of peripheral blood PMN was not reduced by intravenous or subcutaneous $InsP_6$ treatment (not shown). In a closely related murine model, zymosan-induced peritoneal inflammation (Fig. 6*B*), $InsP_6$ (two intravenous injections of 40 μmol/kg at 40 and 80 min) was found to be as effective as in thioglycollate-induced

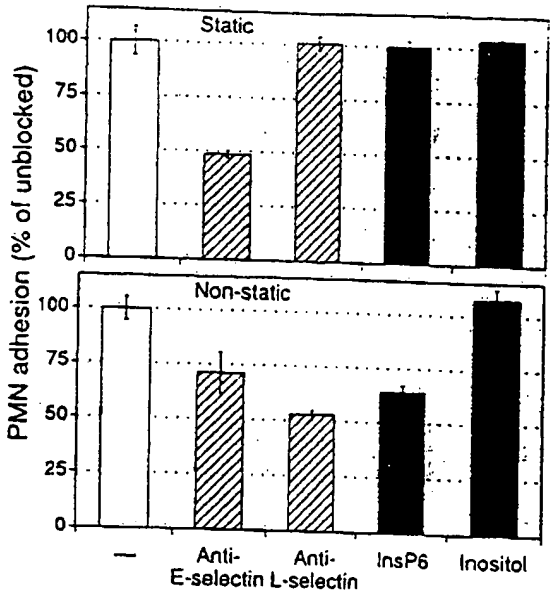

FIG. 5. **Effect of $InsP_6$, inositol, anti-L-selectin, and anti-E-selectin monoclonal antibodies on the adhesion of isolated human PMN to activated endothelial monolayers.** PMN adhesion assay was performed under static (*upper panel*, nonrotating platform) and nonstatic conditions (*lower panel*, rotatory platform, 64 rpm) as described in the text. Inositol and $InsP_6$ concentration was 500 μM. Previous experiments indicated that maximal blocking activity was attained between 250 and 500 μM for $InsP_6$, while inositol was inactive up to 10 mM. Anti-L-selectin antibody (LAM 1.3) was used at a dilution of 1:3000, and anti-E-selectin antibody (H18/7) was used at 10 μg/ml. Data represents the mean ± S.E. of three separate experiments. Unblocked PMN adhesion to activated endothelial monolayers under static and nonstatic conditions ranged from 1800 to 2400 cells/mm^2; unblocked adhesion to unactivated endothelium was less than 50 cells/mm^2.

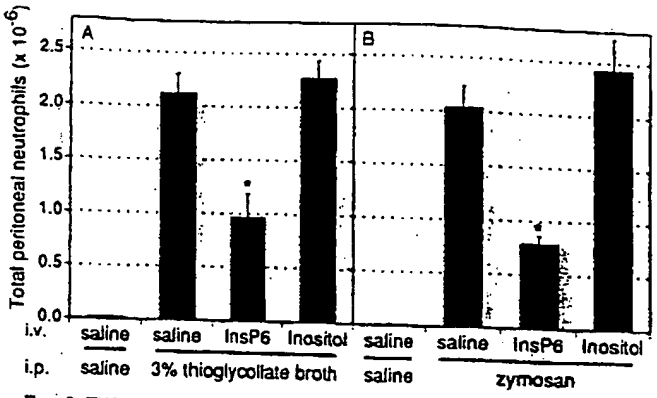

FIG. 6. **Effect of $InsP_6$ on neutrophil accumulation in the peritoneal cavities of mice in two models of acute peritoneal inflammation.** Mice were injected intraperitoneally with 1 ml of 3% thioglycollate broth (Fig. 6*A*) or with 1 ml of saline containing 0.5 mg/ml of zymosan (Fig. 6*B*). Control animals were injected intraperitoneally with 1 ml of sterile pyrogen-free saline. After 40 and 80 min the animals received intravenous injections of 0.2 ml of saline with or without $InsP_6$ (total of 80 μmol/kg = 73 mg/kg) or inositol (400 μmol/kg = 72 mg/kg). Mice were sacrificed after 120 min, and the PMN content of the peritoneal washings was evaluated. *Bars* represents the mean ± S.D. of three separate experiments. Experimental groups contained 5–7 animals/experiment; negative control group (saline intraperitoneally), contained 2 animals/experiment. *Asterisks* indicate statistically significant ($p < 0.01$, Student's *t* test) decrease in the number of PMN recovered in the peritoneal lavage compared with the positive control group (injected intraperitoneally with thioglycollate or zymosan and intravenously with saline). Peripheral blood PMN and platelet counts were similar between $InsP_6$- and saline-treated groups (not shown). Endotoxin content of injected solutions was less than 0.1 endotoxin units/ml. Treatment of animals with intravenous injections of endotoxin (up to 1 endotoxin unit/ml in saline) did not reduce the number of PMN recovered in the peritoneal washings (not shown).

inflammation (61 ± 4% inhibition, 3 experiments). $InsS_6$ at the same dose did not reduce PMN accumulation in these murine models. However, higher doses (two intravenous injections of 200 μmol/kg) were partially effective yielding a 33 ± 13% inhibition in the thioglycollate model and a 31 ± 11% in the zymosan model (1 experiment each, $p < 0.05$ in both experiments). In a rat model of lung inflammation induced by intratracheal injection of endotoxin (44), $InsP_6$ (two intravenous injections of 20 μmol/kg at 2 and 4 h) reduced the number of PMN recovered in the BAL fluid (56 ± 9% inhibition, $p < 0.01$, 2 experiments). The number of peripheral blood PMN was similar in $InsP_6$- and saline-treated animals (not shown).

DISCUSSION

Inositol polyanions were found to be effective inhibitors of L- and P-selectin *in vitro*. Notably, solution-phase $InsP_6$ and $InsP_5$ blocked the binding of purified L-selectin-Ig fusion protein to BSA-sLe^x at a concentration of 1–2 μM. By comparison, solution-phase sLe^x, a known ligand for this selectin, is far less potent, requiring concentrations in excess of 5 mM to achieve comparable blocking (2, 19, 29). Inositol polyanions were also shown to block P-selectin but not E-selectin interactions with BSA-sLe^x. It has been demonstrated previously that solution-phase sLe^x is a relatively good blocker of E-selectin (IC_{50}, ~750 μM) (6). The inhibitory effects of inositol phosphates on L- and P-selectin appear to depend on the number of phosphate groups. $InsP_6$ and $InsP_5$ were most active, $InsP_3$ showed less activity, while $InsP_1$ and inositol itself showed no activity at concentrations up to 5 mM. In preliminary experiments, additional synthetic isomers of $InsP_3$ showed different inhibitory activity (not shown) suggesting the possibility that the position of the phosphate groups in the inositol ring may be important. Other polyphosphates including glucose 2.3.4.6-tetrakisphosphate, 2,3-bisphosphoglycerate, sodium trimetaphosphate, and sodium tripolyphosphate were inactive up to concentrations of 2 mM. Interestingly, the inhibitory effects of inositol polyanions on L- and P-selectin but not E-selectin parallel the recently described effects of heparin, a polysulfated polysaccharide (6, 21, 22, 29). In particular, both high molecular weight heparin (6, 21, 22) and heparin oligosaccharides (29) have been shown to inhibit the function of L- and P-selectin but not E-selectin. In separate studies, endothelial heparin-like molecules have been suggested as ligands for L-selectin (28). Although several proteins are able to bind inositol polyanions, their affinities for the different polyanions vary. For example, hemoglobin (48) and acidic fibroblast growth factor (49, 50) bind $InsP_6$ more avidly than $InsS_6$, whereas β-adrenergic receptor kinase is inhibited more effectively by $InsS_6$ than by $InsP_6$ (51). In our studies, $InsP_6$ and $InsP_5$ are substantially more active than $InsS_6$ in blocking L- and P-selectin.

Recent studies have provided key insights regarding the carbohydrate recognition domains of C-type lectins, including the selectins. In particular, crystallographic studies on the mannose-binding protein (a C-type lectin homologous to selectins) have revealed that two calcium ions associated with the lectin domain participate in ligand binding (52). In addition, directed mutagenesis and monoclonal antibody mapping of E- and P-selectin have suggested that certain basic amino acids within the lectin domains are important for sLe^x binding (53–55). Inositol polyanions are known to bind basic amino acids in several proteins (56) including hemoglobin (48, 57). It is interesting to speculate that the array of negative charge imparted by phosphate or sulfate groups could facilitate the binding of inositol polyanions to basic amino acids within the selectins. It is also possible that the binding of inositol polyanions to calcium ions within the lectin domain could contribute to their ability to alter selectin function.

Selectins are thought to play a critical role in inflammation by controlling the initial attachment of leukocytes to activated vascular endothelium (58, 59). Previous studies in mice have suggested that L- and P-selectin are involved in the early stages of thioglycollate-induced peritoneal inflammation (45–47). PMN recruitment in this model can be reduced by treatment of the animals with the anti-murine L-selectin monoclonal antibody (Mel-14) (45) or with L-selectin-Ig fusion proteins (46). In addition, P-selectin-deficient mice generated by embryonic stem cell gene inactivation displayed a reduced and delayed appearance of PMN in the peritoneal cavity in response to thioglycollate (47). By using thioglycollate-induced peritoneal inflammation, we showed that $InsP_6$, $InsP_5$, and $InsS_6$, injected intravenously or subcutaneously, can effectively reduce PMN accumulation in the peritoneal cavity of thioglycollate-stimulated animals. The anti-inflammatory activity of $InsP_6$ was also shown in zymosan-induced peritoneal inflammation in mice and endotoxin-induced lung inflammation in rats. Recent observations indicate that oligosaccharide ligands of the selectins are effective blockers of inflammatory responses (16, 18, 29, 60). The present data demonstrate that inositol polyanions, which are low molecular weight noncarbohydrate structures, can also reduce experimental inflammatory responses.

$InsP_6$ and $InsP_5$ are found in substantial quantities in most mammalian cells (61–67), where they may act as precursors of several inositol phosphates thought to be involved in intracellular signaling (68–70). In addition, $InsP_6$ is an abundant constituent of many plant seeds and is found in a variety of foods (56). The possibility that these naturally occurring molecules act to suppress inflammatory or immunological responses deserves attention. In addition to blocking the selectins, inositol polyanions have been shown to possess anti-oxidant activity (71) that could enhance their ability to protect against the tissue damage associated with inflammation (72, 73). It is anticipated that inositol polyanions or their derivatives may prove useful as therapeutic agents in the treatment of a variety of human inflammatory diseases.

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Monospecific and Common Glycoprotein Ligands for E- and P-Selectin on Myeloid Cells

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EXHIBIT

B

Abstract. E- and P-selectin are inducible cell adhesion molecules on endothelial cells, which function as Ca^{2+}-dependent lectins and mediate the binding of neutrophils and monocytes. We have recently identified a 150-kD glycoprotein ligand for E-selectin on mouse myeloid cells, using a recombinant antibody-like form of mouse E-selectin. Here, we report that this ligand does not bind to an analogous P-selectin fusion protein. Instead, the chimeric P-selectin-IgG protein recognizes a 160-kD glycoprotein on the mouse neutrophil progenitor 32D cl 3, on mature mouse neutrophils and on human HL60 cells. The binding is Ca^{2+}-dependent and requires the presence of sialic acid on the ligand. This P-selectin-ligand is not recognized by E-selectin. Removal of N-linked carbohydrate side chains from the 150-kD and the 160-kD monospecific selectin ligands abolishes the binding of both ligands to the respective selectin. Treatment of HL60 cells with Peptide:N-glycosidase F inhibited cell binding to P- and E-selectin.

In addition, glycoproteins of 230 and 130 kD were found on mature mouse neutrophils, which bound both to E- and P-selectin in a Ca^{2+}-dependent fashion. The signals detected for these ligands were 15–20-fold weaker than those for the monospecific ligands. Both proteins were heavily sialylated and selectin-binding was blocked by removal of sialic acid, but not by removal of N-linked carbohydrates. Our data reveal that E- and P-selectin recognize two categories of glycoprotein ligands: one type requires N-linked carbohydrates for binding and is monospecific for each of the two selectins and the other type binds independent of N-linked carbohydrates and is common for both endothelial selectins.

THE selectins form a family of three structurally related cell adhesion molecules which mediate early events in the binding of leukocytes to the endothelial lining of blood vessels (Lasky, 1992; Vestweber, 1992). Two of them, E- and P-selectin, are expressed on endothelial cells where they support the binding of neutrophils, monocytes and subsets of lymphocytes (Bevilacqua et al., 1987, 1989; Geng et al., 1990; Picker et al., 1991*a*; Shimizu et al., 1991; Moore and Thompson, 1992). The third, L-selectin, is found on almost all types of leukocytes (Siegelman et al., 1989; Lasky et al., 1989; Tedder et al., 1990) and is also involved in the migration of neutrophils into sites of inflammation (Lewinsohn et al., 1987; Watson et al., 1991). In addition, L-selectin mediates lymphocyte homing into lymph nodes (Gallatin et al., 1983; Hamann et al., 1991) during lymphocyte recirculation.

The selectins are the only family of cell adhesion molecules whose members function as Ca^{2+}-dependent lectins (Hynes and Lander, 1992). All three selectins were shown to bind to the tetrasaccharide sialyl Lewis^x (sLe)[1] (Phillips et al., 1990; Polley et al., 1991; Foxall et al., 1992). Other carbohydrate compounds like the stereoisomer sialyl Lewis^a (Berg et al., 1991*a*; Handa et al., 1991; Tyrrell et al., 1991; Berg et al., 1992) or derivatives of both compounds which carry a SO_3^--group instead of sialic acid (Yuen et al., 1992; Green et al., 1992) bind as well or even better. A derivative of sialyl Lewis^a conjugated to neoglycoproteins was recently demonstrated to be 36-fold more active in E- and P-selectin binding than underivatized, protein-conjugated sLe^x (Nelson et al., 1993). However, the detailed carbohydrate structure of the physiological ligands is still unknown. Most likely, the physiological ligands of the selectins are glycoproteins.

The first glycoprotein ligands for which binding to a selectin was directly demonstrated were identified using an antibody-like L-selectin fusion protein as an affinity probe. With this approach, a 50-kD mucin-like, soluble glycoprotein (GlyCAM-1) and a 90-kD membrane glycoprotein (Sgp90 or

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1. *Abbreviations used in this paper*: CB, complement-binding domains; CP, clostridium perfringens; endo F, endoglycosidase F; PNGase F, Peptide:N glycosidase F; (sLe^x), tetrasaccharide sialyl Lewis^x.

CD34) were isolated from lymph node endothelium (Imai et al., 1991; Lasky et al., 1992; Baumhueter et al., 1993). Both ligands were also recognized by the monoclonal antibody MECA79 which defines the vascular addressin for peripheral lymph nodes (Berg et al., 1991*b*; Streeter et al., 1988). Based on cell adhesion assays, E- and P-selectin have also been described as counterreceptors for the neutrophil type L-selectin (Kishimoto et al., 1991; Picker et al., 1991*b*). However, the affinity of this interaction is too low to directly demonstrate the binding of L-selectin to purified P-selectin or an antibody-like E-selectin fusion protein (Moore et al., 1992; Levinovitz et al., 1993).

Instead, using purified P-selectin from human platelets, a glycoprotein ligand of 120 kD molecular weight under reducing conditions and of 250 kD under non-reducing conditions was identified on human neutrophils and the monocytic cell line HL60 (Moore et al., 1992). With the help of an antibody-like E-selectin fusion protein, we have recently isolated a 150-kD glycoprotein ligand for E-selectin from mouse neutrophils and the mouse neutrophilic progenitor 32D cl 3 (Levinovitz et al., 1993). In addition, a minor component of 250 kD molecular weight was detected on mature mouse neutrophils.

From the published data, it is difficult to compare the P- and E-selectin ligands since different cells were analyzed and the affinity probes, the isolation procedures and the labeling techniques differed significantly. Here, we have directly approached the question whether E- and P-selectin recognize different or similar ligands. Using analogous antibody-like fusion proteins for mouse E- and P-selectin, we have performed affinity isolation experiments on detergent extracts of mouse neutrophils, the mouse neutrophilic progenitor 32D cl 3 and the human monocytic cell line HL60. We have found two glycoproteins of which each is a specific ligand for only one of the two endothelial selectins. Two additional, more weakly detectable glycoproteins were found which were common ligands for both selectins.

Materials and Methods

Cells

The neutrophilic progenitor 32D cl 3 (Valtieri et al., 1987; Migliaccio et al., 1989) provided by Dr. Rovera (Wistar Institute, Philadelphia) was grown in DMEM with 20% FCS, supplemented with 10% conditioned medium of WEHI-3B cells (as source for IL-3). The human monocytic cell line HL60 was obtained from Amer. Type Culture Collection (Rockville, MD) and cultured in DMEM containing 10% FCS. J558L cells secreting either the P-selectin-IgG, the E-selectin-IgG, or CD4-IgG fusion protein were grown in DMEM supplemented with 10% BMS (Seromed, Berlin, Germany). Mouse neutrophils (polymorphonuclear granulocytes) were freshly isolated from the femurs of 10 wk old NMRI mice as described (Levinovitz et al., 1993).

For the isolation of human neutrophils, 10 ml of heparinized blood was mixed with 20 ml of sterile HBSS, underlayed with 10 ml of Histopaque 1077 (Sigma Chem. Co., St. Louis, MO) and centrifuged for 30 min at 2,300 rpm. Pelleted cells were washed once in RPMI containing 2% FCS and resuspended in HBSS (original blood volume). 20% (vol/vol) of a 6% Dextran 500 (Pharmacia LKB Biotechnology, Piscataway, NJ) solution in PBS was added and the cell suspension was loaded into a vertical syringe, needle upright. Erythrocytes were allowed to settle for 20 min. Leukocytes were expelled and washed twice in RPMI.

Antibodies

The anti-mouse P-selectin rabbit antiserum was raised against the purified P-selectin-immunoglobulin chimeric protein as described (Hahne et al., 1993). Specific antibodies were affinity purified on the fusion protein conjugated to CNBr-Sepharose (Pharmacia Uppsala, Sweden). Antibodies against the Fc part of human IgG were removed by a second CNBr-Sepharose column with conjugated human IgG (Sigma Immunochemical, St. Louis, MO).

The rat IgM mAb 21KC10 against mouse E-selectin was recently described (Hahne et al., 1993) as was the rat IgM mAb 2&AG6 which had been raised against the E-selectin-IgG chimeric protein and recognizes the Fc-region of human IgG1 (Levinovitz et al., 1993).

Cell Adhesion Assay with Plastic-coated P- and E-selectin-IgG

96 well microtiter plates (Falcon, Heidelberg, Germany) were coated with 20 μg/ml P-selectin IgG, E-selectin-IgG, or CD4-IgG in HBSS (Biochrom, Berlin, Germany) and subsequently blocked with 10% FCS in HBSS. 0.5–1 × 10^6 cells in 200 μl were added to each well and allowed to bind for 20 min at 7°C under mild rotation (50 rpm). Unbound cells were removed by flicking out the plates and washing five times with HBSS. Bound cells were fixed with 2% glutaraldehyde in HBSS for 1 h at 7°C, followed by one additional wash with HBSS. Bound cells were quantitated by counting the cells under the microscope in 10 randomly chosen areas for each well. Each determination was done for four wells. Antibody inhibition was tested by preincubating the coated and blocked wells with antibodies at the indicated concentrations for 45 min at 37°C. Unbound antibodies were washed away before cells were added.

Enzyme treatments of intact HL60 cells were performed in 1 ml DMEM without FCS at a cell density of 5 × 10^5/ml for 1 h at 37°C with 1 U endoglycosidase F (endo F) or 2 U Peptide:N glycosidase F (PNGase F) (both from Boehringer, Mannheim, Germany) or 20 μl O-sialoglycoprotease from *Pasteurella hemolytica* (Cedarlane, Hornby, Ontario, Canada, purchased from Camon, Wiesbaden, Germany). The lyophilated enzyme, containing Hepes buffer salts and bovine serum proteins, was reconstituted with water to a final protein concentration of 2.5 mg/ml with a specific activity of 5 mg human glycophorin A cleaved per mg protein per hour at 37°C. For controls, cells were incubated in the same way in the absence of the enzymes. To control whether the amount of PNGase F and endo F were sufficient, enzyme treatment of cells was done in the presence of 20 μg of an L-selectin-IgG fusion protein. This protein was affected by the glycosidases in the presence of the cells as efficiently as in the absence of the cells.

Selectin-Immunoglobulin Chimeric Proteins

The construction as well as the production of the P- and E-selectin-IgG chimeric proteins has been described (Hahne et al., 1993). In both fusion proteins, the selectin part includes the signal sequence, the lectin domain, the EGF-like repeat and the first two "complement-binding (CB)" domains, except for the last two amino acids of the second CB-domains.

Affinity Isolation of the Metabolically Labeled P- and E-Selectin Ligands

HL 60 and 32D cl 3 cells were labeled for 4 h with 400 μCi [^{35}S]methionine and 200 μCi [^{35}S]cysteine in 1 ml medium (5 × 10^6 cells/ml) in MEM without methionine and cysteine (Gibco/BRL, Karlsruhe, Germany), supplemented with 10% FCS which had been dialyzed against PBS. 4 × 10^7 freshly isolated PMNs from mouse bone marrow were labeled in 500 μl of the same medium with 600 μCi [^{35}S]methionine and 600 μCi [^{35}S]-cysteine for 4 h.

Labeled cells were lysed at a density of 0.5–3 × 10^7 cells/ml in lysis buffer (3% CHAPS; 50 mM Tris-HCl, pH 7.4; 150 mM NaCl, 1 mM $CaCl_2$, 1 mg/ml Ovalbumin, 1 mM PMSF) for 10 min, insoluble material was pelleted at 10,000 *g* for 10 min; these and all subsequent steps were performed at 4°C. The lysate was preincubated for 30 min with 50 μl of packed protein A-Sepharose. After removal of the Sepharose beads, carrying unspecifically bound proteins, the cell extract was aliquoted and fractions routinely corresponding to 10^6–10^7 cells were incubated with 10–20 μl of protein A-Sepharose preloaded overnight with 10–20 μg of P-selectin-IgG, E-selectin-IgG, or CD4-IgG. After an incubation period of 4 h, the resin was washed five times with washing buffer (0.05% Triton X-100; 50 mM Tris-HCl pH 8.5; 400 mM NaCl; 1 mM $CaCl_2$) and two more times with a similar washing buffer, lacking $CaCl_2$ and containing 150 mM instead of 400 mM NaCl. Elution was done twice with 60 μl 3 mM EDTA in 50 mM sodium acetate pH 5.2; 0.05% Triton X-100. Eluted proteins were sepa-

rated by SDS-PAGE and detected either by fluorography using Kodak X-AR X-ray films (Kodak, Stuttgart, Germany) or by analysis in a Fujix BAS 1000 Bio-Imaging-Analyser (Fuji, Japan).

In cross-precipitation experiments the cell extract of metabolically labeled mouse neutrophils (see above) was incubated with the P-selectin-IgG affinity matrix, the EDTA eluate was added to 500 μl 50 mM Tris pH 7.4, 150 mM NaCl, 4 mM $CaCl_2$, 1% Triton X-100 and equal aliquots were again incubated with P-selectin-IgG or E-selectin-IgG affinity matrices. Bound proteins were again eluted with EDTA and analyzed as described above.

Sialidase, Endoglycosidase F, and PNGase F Treatment of Selectin Ligands

All enzymes were purchased from Boehringer (Mannheim, Germany). For sialidase treatment, the P-selectin ligands were eluted from the affinity matrix in 3 mM EDTA; 50 mM sodium acetate pH 5.2; 0.05% Triton X-100. 50 μl EDTA-eluate corresponding to 1.5×10^7 [^{35}S]methionine [^{35}S]cysteine labeled neutrophils or to 2×10^6 HL60 cells were digested either with 500 mU of neuraminidase from Arthrobacter ureafaciens, or 20 mU neuraminidase from New Castle disease virus or 100 mU neuraminidase from Clostridium perfringens overnight at 37°C. Mock treatment was done under identical conditions in the absence of the enzyme. 40% of the digest was set aside for PAGE while 60% were subjected to reprecipitation by overnight incubation with P-selectin-IgG protein A-Sepharose after adding 500 μl of 1% Triton X-100; 50 mM Tris-HCl pH 7.4; 150 mM NaCl; 4 mM $CaCl_2$. Subsequent washing and elution was done as described above. For digestion with endo F and with PNGase F, the selectin-ligands were affinity isolated from labeled HL60 cells, 32D cl 3 cells or neutrophils as described above using E- or P-selectin-IgG and eluted with EDTA. 50 μl EDTA-eluate corresponding to 2×10^6 HL60 or 32D cl 3 cells or 1.5×10^7 neutrophils was supplemented with 0.05% Triton X-100 and digested overnight at 37°C without (control) or with 0.5 U (10 μl) of endo F or with 2 U (10 μl) of PNGase F. Reprecipitation was done as described above.

O-sialoglycoprotease Treatment of Selectin Ligands

Ligands were affinity-isolated from [^{35}S]methionine [^{35}S]cysteine labeled HL60 cells, 32D cl 3 cells or mature mouse neutrophils with E-selectin-IgG or P-selectin-IgG and eluted with 60 μl 3 mM EDTA, 50 mM ammonium acetate pH 7.0. The eluate was substituted with 60 μl 100 mM Hepes pH 7.4 split into halves and one half was incubated with 1 μl O-sialoglycoprotease (1 μl cleaved 12.5 μg glycophorin A, per h at 37°C) and the other half without the enzyme for 1 h at 37°C. Proteins were analyzed by PAGE and fluorography or phosphorimaging.

Results

E-Selectin-IgG and P-Selectin IgG Specifically Support the Binding of Myeloid Cells

We have recently identified a 150-kD glycoprotein ligand for E-selectin on mouse myeloid cells using a mouse E-selectin-IgG fusion protein (Levinovitz et al., 1993) as affinity probe. We have now used an analogous mouse P-selectin-IgG fusion protein to identify P-selectin ligands and to directly compare the ligands of both selectins. To test whether the P-selectin-IgG fusion protein functions in a selectin-specific way, we analyzed whether it supports the binding of myeloid cells when coated to plastic. The myeloid cells used in this assay were the mouse neutrophilic progenitor 32D cl 3, the human monocytic cell line HL60 and mature mouse neutrophils. All three cell types bound to P-selectin-IgG as well as to E-selectin-IgG while no binding was observed to CD4-IgG (Fig. 1). Binding was dependent on the presence of Ca^{2+} since it was completely blocked by EDTA (Fig. 1). Affinity purified, polyclonal antibodies to P-selectin blocked cell binding to P-selectin-IgG but not to E-selectin-IgG. Similarly, the mAb 21KC10 against mouse E-selectin only blocked cell binding to E-selectin-IgG but not to P-selectin-IgG. A control IgM mAb (28AG6) against the Fc-part of both fusion proteins did not block cell binding. We conclude that similar to the E-selectin-IgG chimeric protein, the P-selectin-IgG fusion protein also binds via its selectin part in a Ca^{2+}-dependent way to myeloid cells. Binding occurs across the species barrier between man and mouse.

E-Selectin-IgG and P-Selectin-IgG Bind to Two Different Glycoprotein Ligands on Myeloid Cells

The selectin fusion proteins were bound to protein-A Sepharose and used as affinity matrix to identify and isolate ligands from detergent extracts of the mouse neutrophilic progenitor 32D cl 3. Cells were metabolically labeled with [^{35}S]methionine [^{35}S]cysteine and aliquots of a detergent extract of these cells were incubated with the affinity-matrices. Specifically bound proteins were eluted with EDTA. As expected, the 150-kD E-selectin ligand was eluted from the E-selectin-IgG matrix (Fig. 2 *A*). This protein, however, did not bind to P-selectin-IgG. Instead, a single protein, migrating at an apparent molecular weight of 160 kD under non-reducing conditions was eluted from the P-selectin-IgG matrix (Fig. 2 *A*). Neither the E-selectin ligand nor the 160-kD P-selectin ligand bound to CD4-IgG.

Affinity isolation experiments on cell lysates of [^{35}S]methionine/[^{35}S]cysteine labeled human HL60 cells gave similar results. The two isolated glycoproteins had the same molecular weight as the mouse ligands and each of them was only bound by one of the two selectin fusion proteins (Fig. 2 *B*). Thus, the two endothelial selectins each recognize a different glycoprotein ligand on myeloid cells. These ligands were recognized on mouse as well as on human cells.

In contrast to the E-selectin ligand which migrates at 130 kD apparent molecular weight under non-reducing conditions and at 150 kD under reducing conditions the P-selectin ligand could only be detected in its non-reduced form. When the EDTA-eluted 160-kD protein isolated from either mouse or human cell lines was boiled in PAGE-loading buffer in the presence of DTT, the protein was no longer detectable on a 10% polyacrylamide gel. Instead, a signal at the top of the stacking gel was found, indicating that reduction of the protein caused the formation of insoluble aggregates (not shown). However, when the non-reduced 160-kD proteins, isolated from both cell lines, were excised from a polyacrylamide gel after the first electrophoresis and reelectrophoresed on a second gel under reducing conditions, both proteins migrated at an apparent molecular weight of 80 kD (Fig. 3). This indicates, that the 160-kD P-selectin ligand may be a disulfide-linked dimer.

The 160-kD P-Selectin Ligand Requires Both Sialic Acid and N-linked Carbohydrates for the Binding to P-Selectin

Since selectins bind to carbohydrate structures, we tested whether the 160-kD ligand is glycosylated and whether carbohydrates would be involved in the binding to P-selectin. The 160-kD protein was isolated from [^{35}S]methionine/[^{35}S]cysteine labeled HL60 cells as described above and treated with three different sialidases at 37°C overnight. Two of them, the neuraminidases from Arthrobacter ureafaciens and from New Castle disease virus, did not affect the elec-

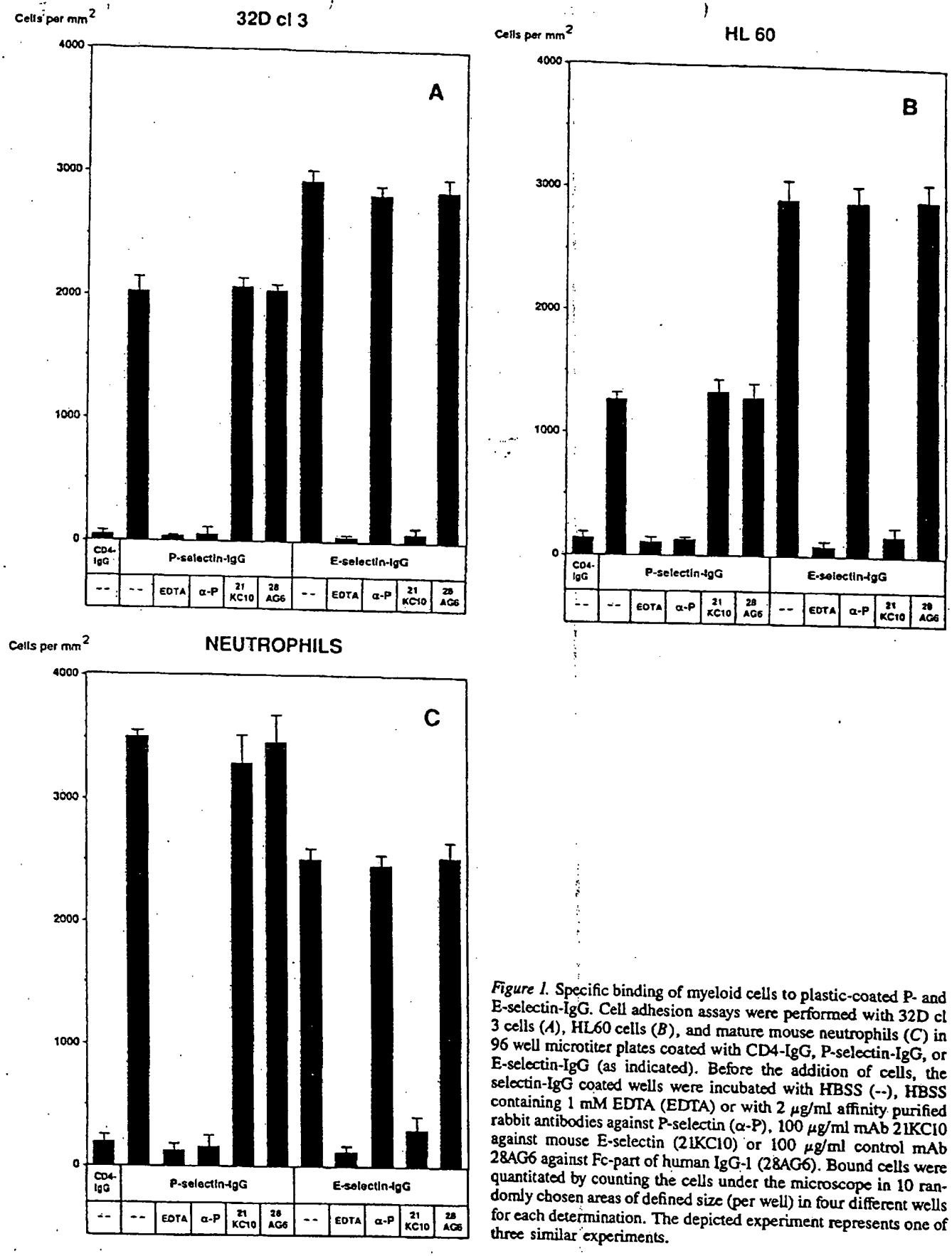

Figure 1. Specific binding of myeloid cells to plastic-coated P- and E-selectin-IgG. Cell adhesion assays were performed with 32D cl 3 cells (*A*), HL60 cells (*B*), and mature mouse neutrophils (*C*) in 96 well microtiter plates coated with CD4-IgG, P-selectin-IgG, or E-selectin-IgG (as indicated). Before the addition of cells, the selectin-IgG coated wells were incubated with HBSS (--), HBSS containing 1 mM EDTA (EDTA) or with 2 μg/ml affinity purified rabbit antibodies against P-selectin (α-P), 100 μg/ml mAb 21KC10 against mouse E-selectin (21KC10) or 100 μg/ml control mAb 28AG6 against Fc-part of human IgG-1 (28AG6). Bound cells were quantitated by counting the cells under the microscope in 10 randomly chosen areas of defined size (per well) in four different wells for each determination. The depicted experiment represents one of three similar experiments.

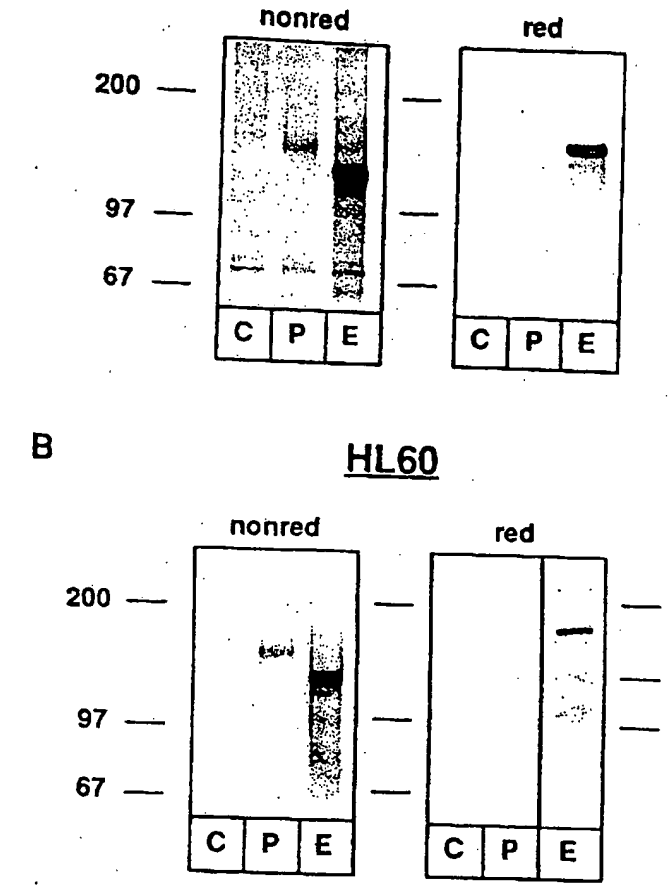

Figure 2. Affinity isolation of two different glycoprotein ligands of which each is specific for either P- or E-selectin. 32D cl 3 cells (*A*) and HL60 cells (*B*) were metabolically labeled with [^{35}S]methionine/[^{35}S]cysteine and detergent extracts were incubated either with immobilized CD4-IgG (*C*), P-selectin-IgG (*P*), or with E-selectin-IgG (*E*). Specifically bound proteins were eluted with EDTA and electrophoresed on a 6% polyacrylamide gel under non-reducing (*non-red*) or reducing (*red*) conditions, except for panel *B*, last lane, where the proteins were separated on an 8% gel. Molecular mass markers (in kD) are indicated.

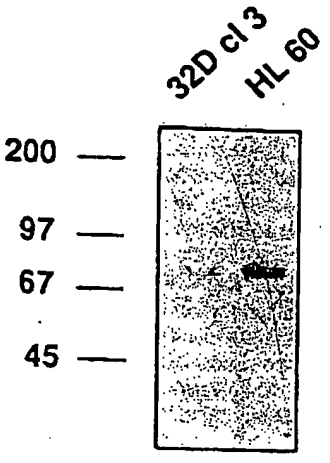

Figure 3. The 160-kD P-selectin ligand is cleaved to an 80-kD monomer by reduction. The 160-kD P-selectin ligand was affinity-isolated from [^{35}S]methionine/[^{35}S]cysteine labeled 32D cl 3 cells or HL60 cells, separated on a polyacrylamide gel under non-reducing conditions, excised from the gel and reelectrophoresed under reducing conditions on a second 8% polyacrylamide gel giving rise to proteins migrating at 80 kD apparent molecular weight. Labeled proteins were visualized by phosphorimaging. Molecular mass markers (in kD) are indicated on the left.

trophoretic mobility of the ligand (not shown). However, the neuraminidase from Clostridium perfringens (CP) clearly reduced the apparent molecular weight of the ligand (Fig. 4 *A*, lane *3*), although also in this case ~50% of the ligand molecules were unaffected. If aliquots of the mock and the

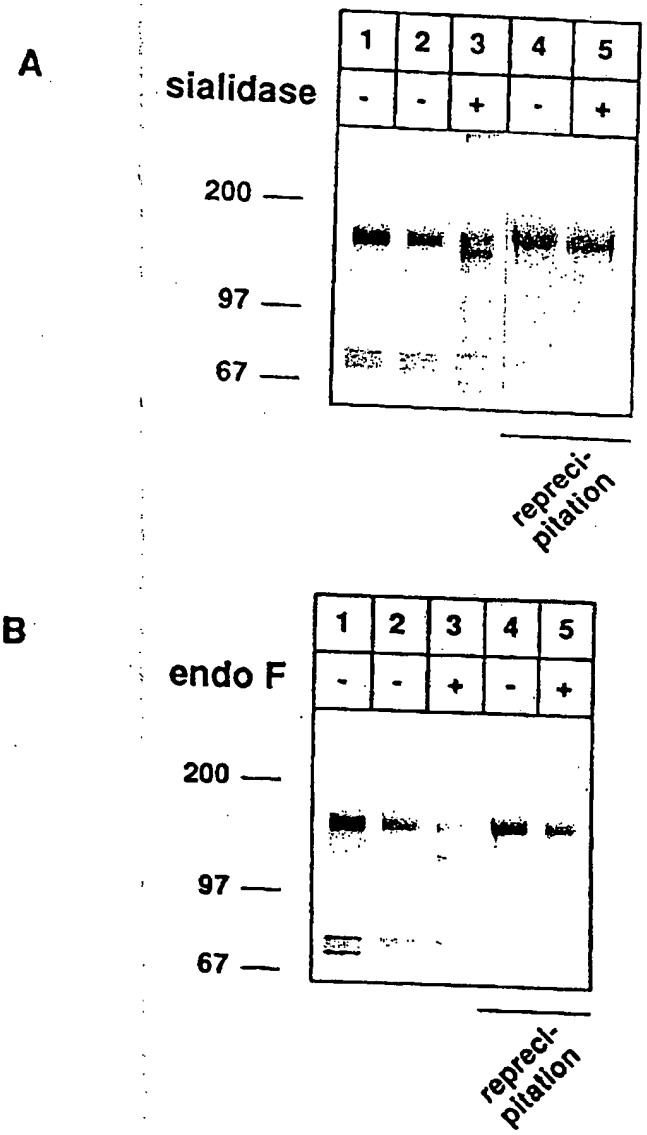

Figure 4. The 160-kD P-selectin ligand requires both sialic acid as well as N-linked carbohydrates for binding to P-selectin. The 160-kD P-selectin ligand was affinity-isolated from [^{35}S]methionine/[^{35}S]cysteine labeled HL60 cells with immobilized P-selectin-IgG. (*A*) The isolated ligand was either directly electrophoresed (lane *1*) or treated with (lanes *3* and *5*) or without (lanes *2* and *4*) 100 mU of sialidase from Clostridium perfringens overnight at 37°C. 40% of the treated samples were directly electrophoresed (lanes *2* and *3*) and 60% reprecipitated with P-selectin-IgG (lanes *4* and *5*). (*B*) Similar as panel *A*, except that endo F instead of sialidase was used. The ligand molecules which were affected by the two enzymes in their electrophoretic mobility (lane *3* in both panels) were in each case not reprecipitated by P-selectin-IgG (lane *5* in both panels). Proteins were electrophoresed on 6% polyacrylamide gels under non-reducing conditions and labeled proteins were visualized by fluorography. Molecular mass markers (in kD) are indicated on the left.

CP-neuraminidase-treated ligand were reprecipitated with P-selectin-IgG, only the unaffected molecules bound the fusion protein while binding of those molecules which were shifted in molecular weight was completely abolished. Thus, sialic acid on the 160-kD ligand is required for binding to P-selectin.

We tested whether N-linked carbohydrates on the ligand would be relevant for the binding process. Treatment of the isolated, [^{35}S]methionine/[^{35}S]cysteine labeled ligand with endo F (Fig. 4 *B*) and also with PNGase F (not shown) caused a similar reduction of the apparent molecular weight of the ligand (Fig. 4 *B*, lane *3*) although again not all molecules were susceptible to the enzymes. Reprecipitation of ligand molecules from which N-linked carbohydrates had been removed, either with endo F (Fig. 4 *B*) or with PNGase F (not shown), was completely abolished in both cases, while the mock-treated material and also undigested ligand molecules, which had been left intact by endo F, were still reprecipitated normally (Fig. 4 *B*, lane *5*). Thus, oligosaccharides on N-linked carbohydrate side chains of the 160-kD glycoprotein are essential for the binding to P-selectin.

The 150-kD E-Selectin Ligand Also Requires N-linked Carbohydrates for the Binding to E-selectin

We have recently shown that removal of sialic acid from the 150-kD E-selectin-ligand strongly reduces (by 80%) its binding to the E-selectin-IgG fusion protein (Levinovitz et al., 1993). The results with the 160-kD P-selectin-ligand prompted us to test whether the E-selectin ligand would also require N-linked carbohydrates for binding. The 150-kD E-selectin ligand was affinity isolated with E-selectin-IgG from [^{35}S]-methionine/[^{35}S]cysteine labeled 32D cl 3 cells and treated with endo F. This treatment reduced the apparent molecular weight of the isolated ligand (Fig. 5, lane *4*). The shifted form of the ligand could only be reprecipitated with E-selectin-IgG with an efficiency of 20% as compared to the mock-treated sample (Fig. 5, lane *6*). Thus, N-linked carbohy-

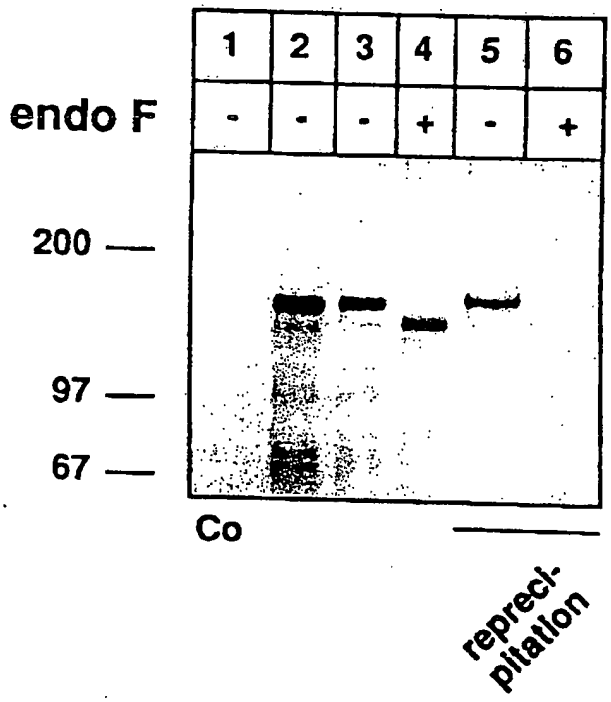

Figure 5. The 150-kD E-selectin ligand requires N-linked carbohydrates for binding to E-selectin. 32D cl 3 cells were metabolically labeled with [^{35}S]methionine/[^{35}S]cysteine and detergent extracts were incubated either with immobilized CD4-IgG (*Co*) or E-selectin-IgG (all other lanes). The isolated 150-kD E-selectin-ligand was directly electrophoresed (lane *2*) or treated with (lanes *4* and *6*) or without (lanes *3* and *5*) 1 U endo F overnight at 37°C. Half of the treated samples were directly electrophoresed (lanes *3* and *4*) or reprecipitated with E-selectin-IgG (lanes *5* and *6*). Electrophoresis was performed under reducing conditions on a 6% polyacrylamide gel and labeled proteins were visualized by fluorography. Scanning of the depicted x-ray film revealed that the endo F treatment decreased the signal of the reprecipitated 150-kD ligand (lane *6*) to 20% when compared with the signal of the mock-treated, reprecipitated ligand (lane *5*). Molecular mass markers (in kD) are indicated on the left.

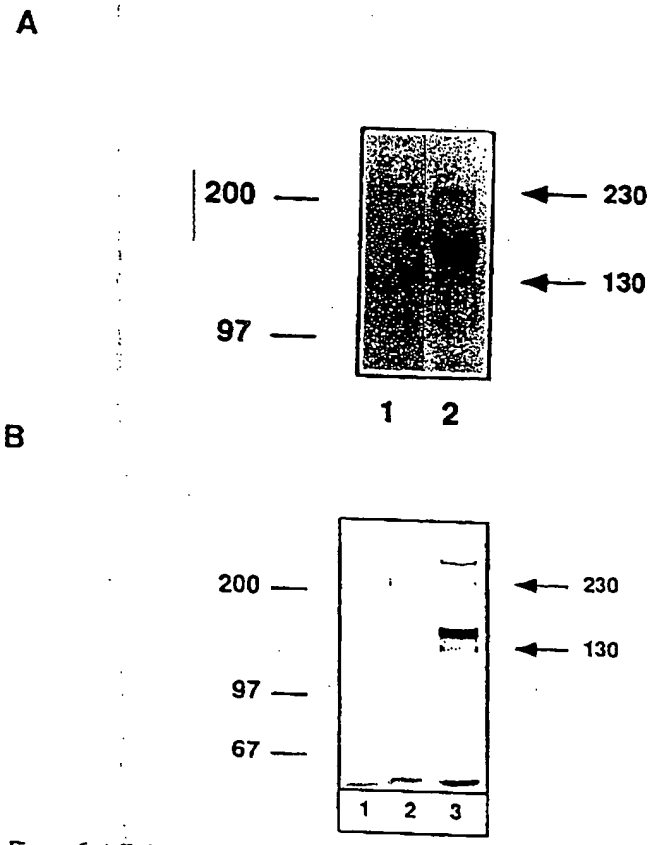

Figure 6. Affinity isolation of monospecific and of common glycoprotein ligands for P- and E-selectin from mouse neutrophils. Mouse neutrophils were metabolically labeled with [^{35}S]methionine/[^{35}S]cysteine and detergent extracts were incubated either with P-selectin-IgG (*A*, lanes *1* and *2*; *B*, lane *2*), E-selectin-IgG (*B*, lane *3*), or CD4-IgG (*B*, lane *1*). Specifically bound proteins were eluted with EDTA and electrophoresed on a 6% polyacrylamide gel under nonreducing (*A*, lane *2*) or reducing (*A*, lane *1*; *B*, lanes *1–3*) conditions. In addition to the monospecific 160-kD P-selectin ligand (non-reduced) and 150-kD E-selectin ligand (reduced), two common ligands of 230 kD and 130 kD apparent molecular weight were identified (marked by arrows on the right) which bind to both selectins. Scanning of the depicted x-ray film revealed that the signals of the two common ligands were 15–20-fold weaker than that of the 150-kD E-selectin ligand and 10–15-fold weaker than the 160-kD P-selectin ligand. Molecular mass markers (in kD) are indicated on the left.

drates on the 150-kD ligand are involved in the binding to E-selectin.

Two Additional Weakly Detectable Ligands on Mature Mouse Neutrophils Bind to Both Endothelial Selectins

Like on the two cell lines described above, we detected the 160-kD P-selectin ligand in affinity isolation experiments with P-selectin-IgG on detergent extracts of [^{35}S]methionine/[^{35}S]cysteine labeled mature mouse neutrophils (Fig. 6 *A*, lane *2*). The neutrophil-derived ligand, like the 160-kD ligands from the two cell lines, was undetectable if the eluted protein was reduced in solution before electrophoresis (Fig. 6 *A*, lane *1*). Again, this protein migrated with an apparent molecular weight of 80 kD if the 160-kD non-reduced form of the protein was excised from a gel, and then reduced before a second electrophoresis (data not shown).

In addition to this protein we found two more ligands which migrated at apparent molecular weights of 230 and 130 kD under reducing as well as under non-reducing conditions (Fig. 6 *A*, lanes *1* and *2*). The signals for these proteins were 10–15-fold weaker than the signal for the 160-kD ligand. Proteins of identical molecular weight were detected in affinity isolation experiments with E-selectin-IgG (Fig. 6 *B*, lane *3*) in addition to the 150-kD E-selectin ligand and the 250-kD ligand, of which the latter was exclusively found on mature mouse neutrophils (Levinovitz et al., 1993). The signals for the 230- and 130-kD proteins were 15–20-fold weaker than the signal for the 150-kD ligand. These two additional proteins had not been detected before with E-selectin-IgG and were discovered now due to improving the labeling conditions of neutrophils and due to exchanging the detergent Triton X-100 by CHAPS (see Materials and Methods section). On human neutrophils, isolated from peripheral blood, E- and P-selectin-ligands of similar molecular weight were identified as on mouse neutrophils (data not shown).

To test directly whether the 230/130-kD ligand pairs recognized by each selectin are indeed identical, we affinity-isolated the proteins with P-selectin-IgG, and reincubated equal aliquots (after compensating the EDTA with Ca^{2+}) with P-selectin-IgG or E-selectin-IgG, respectively. As shown in Fig. 7, the affinity-purified 230/130-kD ligand pair was equally well recognized by both selectin fusion proteins (lanes *3* and *4*), demonstrating that this pair of proteins represents common ligands for both endothelial selectins.

In different affinity isolation experiments, using P-selectin-IgG as affinity probe, the ratio of the 230- and 130-kD common selectin ligands varied slightly. In most cases, the 130-kD protein was more easily detectable under reducing than under non-reducing conditions, although it was always detectable under both conditions. Because of such variations, we tested whether reduction of the 230-kD protein could give rise to the 130-kD band. The 230-kD protein was electrophoresed under non-reducing conditions, cut from the gel and reelectrophoresed under reducing conditions. Under such conditions, reduction did not influence the migration of the 230-kD protein and did not give rise to a protein of 130 kD apparent molecular weight (data not shown). However, it is possible that this reduction procedure at room temperature was not sufficient to fully reduce the protein.

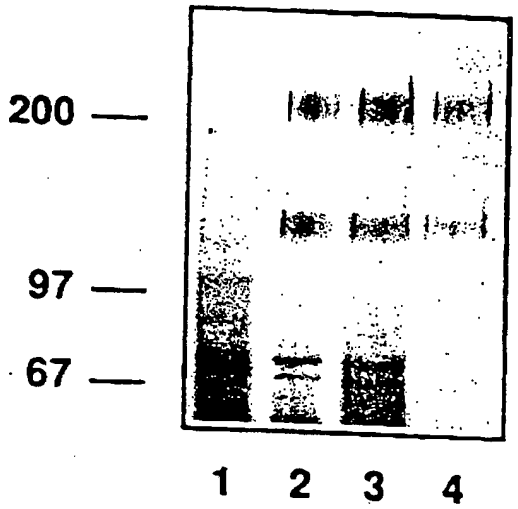

Figure 7. The 230/130-kD ligands recognized by P-selectin are identical with those recognized by E-selectin. Mouse neutrophils were metabolically labeled with [^{35}S]methionine/[^{35}S]cysteine and 20% of the detergent extract was incubated with CD4-IgG (lane *1*) and 80% with P-selectin-IgG. Proteins bound to P-selectin-IgG were eluted by EDTA and aliquots were either directly electrophoresed (lane *2*) or substituted with Ca^{2+} and reprecipitated with P-selectin-IgG (lane *3*) or E-selectin-IgG (lane *4*). The aliquots loaded per lane corresponded to 20% (lanes *1* and *2*) or 30% (lanes *3* and *4*) of the total cell extract. Molecular mass markers (in kD) are indicated on the left.

The 230-kD and the 130-kD Ligands Are Heavily Sialylated and Require Sialic Acid, But No N-linked Carbohydrates for Selectin-Binding

To test whether the two ligands, common to both selectins, contained sialic acid, the metabolically labeled proteins were purified with P-selectin-IgG as described and the EDTA-eluted material was treated overnight at 37°C with sialidase from Arthrobacter ureafaciens. As shown in Fig. 8, this treatment caused a similar increase of the apparent molecular weight of the 230- and the 130-kD ligands while the mock-treatment had no effect. When aliquots of the sialidase and the mock-treated samples were subjected to reprecipitations with P-selectin-IgG affinity beads, binding of the sialidase-treated 230-kD and the 130-kD ligands was completely abolished (Fig. 8 *A*, lane *6*). Thus, sialic acid on the 230-kD and the 130-kD ligands is essential for the binding to P-selectin.

In the same way we tested the effect of endo F-treatment on the binding of the ligands to P-selectin-IgG. The affinity isolated proteins were clearly reduced in apparent molecular weight by treatment with endo F (Fig. 8 *B*, lane *4*), however the endo F-digested molecules were as efficiently reprecipitated with the selectin fusion protein as the mock-treated ligand molecules (Fig. 8 *B*, lane *6*). Similarly, the endo F-treatment did not interfere with the binding of the 230/130-kD ligands to E-selectin-IgG (data not shown). This indicates that in contrast to the two monospecific selectin ligands, N-linked carbohydrates are not essential for selectin-binding of the 230/130-kD glycoproteins.

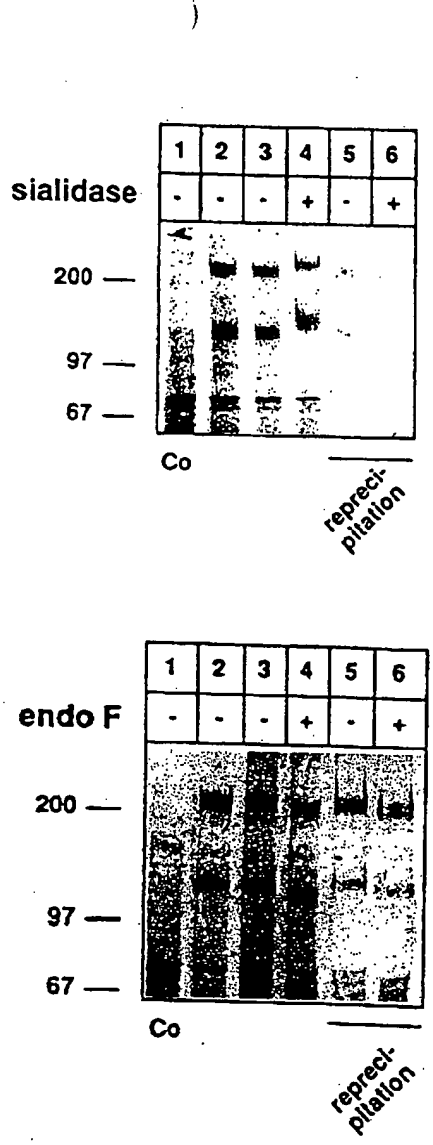

Figure 8. The 230- and 130-kD common selectin-ligands require sialic acid but no N-linked carbohydrates for the binding to P-selectin. Mouse neutrophils were metabolically labeled with [^{35}S]methionine/[^{35}S]cysteine and detergent extracts were incubated either with immobilized CD4-IgG (*Co*) or P-selectin-IgG (all other lanes). (*A*) The isolated 230- and 130-kD ligands were either directly electrophoresed (lane *2*) or treated with (lanes *4* and *6*) or without (lanes *3* and *5*) 1 U of sialidase from Arthrobacter ureafaciens overnight at 37°C. Half of the treated samples were directly electrophoresed (lanes *3* and *4*) or reprecipitated with P-selectin-IgG (lanes *5* and *6*). (*B*) Similar as panel *A*, except that endo F instead of sialidase was used. Electrophoresis was performed under reducing conditions on a 6% polyacrylamide gel and labeled proteins were visualized by phosphorimaging. Molecular mass markers (in kD) are indicated on the left.

The 230/130-kD Ligand-Pair and the 160-kD P-Selectin Ligand, But Not the 150-kD E-Selectin Ligand Are Sensitive to O-sialoglycoprotease

The O-sialoglycoprotease from *Pasteurella hemolytica* was described to specifically cleave O-glycosylated sialoglycoproteins (Sutherland et al., 1992). We have analyzed which of the described E- and P-selectin ligands would be sensitive to this protease. To this end the ligands were isolated from [^{35}S]methionine/[^{35}S]cysteine labeled cells: the 230- and 130-kD ligands were isolated with P-selectin-Ig from mouse neutrophils, the 160-kD P-selectin ligand was isolated in the same way from HL60 cells and the 150-kD ligand was isolated with E-selectin-IgG from 32D cl 3 cells. In each case the EDTA eluates were split into halves and one half was incubated for 1 h at 37°C without and the other half with 2.5 μg of the protein-stabilized enzyme (see Materials and Methods). As shown in Fig. 9, the protease degraded all of the 230-kD/130-kD ligands and 70% of the 160-kD P-selectin ligand molecules while the 150-kD E-selectin ligand was unaffected. This indicates that the 150-kD E-selectin ligand is not heavily O-glycosylated.

PNGase F-Treatment of HL60 Cells Blocks Binding to E- and P-Selectin, While O-sialoglycoprotease-treatment Only Blocks Cell Binding to P-selectin

If the monospecific selectin-ligands, which require N-linked carbohydrates for selectin-binding, indeed represent cell adhesion ligands for the selectins, removal of N-linked carbohydrates from the surface of intact cells should inhibit cell-binding. Therefore, the adhesion of HL60 cells to plastic-coated P- or E-selectin-IgG was tested after treating the cells with PNGase F for 1 h at 37°C. As shown in Fig. 10 *A* binding of HL60 cells to P- as well as to E-selectin was inhibited by this enzyme treatment when compared to mock-treated cells. The same result was observed for endo F-treated cells (not shown). In contrast, treatment of intact HL60 cells

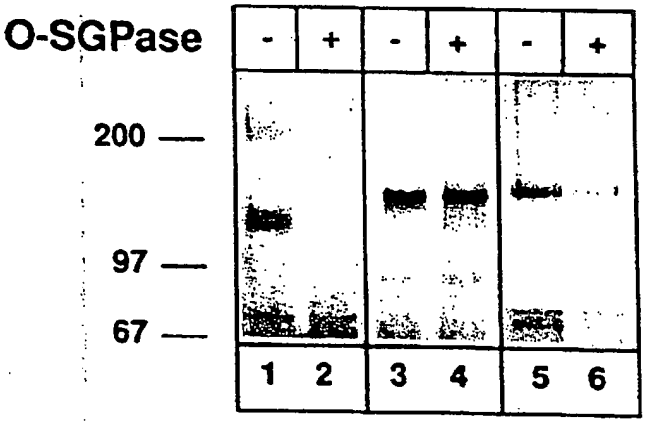

Figure 9. The common but not the monospecific E-selectin ligand is sensitive to O-sialoglycoprotease. Mouse neutrophils (lanes *1* and *2*), 32D cl 3 cells (lanes *3* and *4*) and HL60 cells (lanes *4* and *6*) were metabolically labeled with [^{35}S]methionine/[^{35}S]cysteine and detergent extracts were incubated either with immobilized P-selectin-IgG (lanes *1*, *2*, *5*, and *6*) or with E-selectin-IgG (lanes *3* and *4*). Specifically bound proteins were eluted with EDTA and half of the eluate was mock treated and the other half was treated with O-sialoglycoprotease (O-SGPase) from *Pasteurella hemolytica*. Proteins were electrophoresed on a 6% polyacrylamide gel under reducing (lanes *1–4*) and non-reducing conditions (lanes *5* and *6*) and visualized by phosphorimaging (lanes *1*, *2*, *5*, and *6*) and fluorography (lanes *3* and *4*). Molecular mass markers (in kD) are indicated on the left.

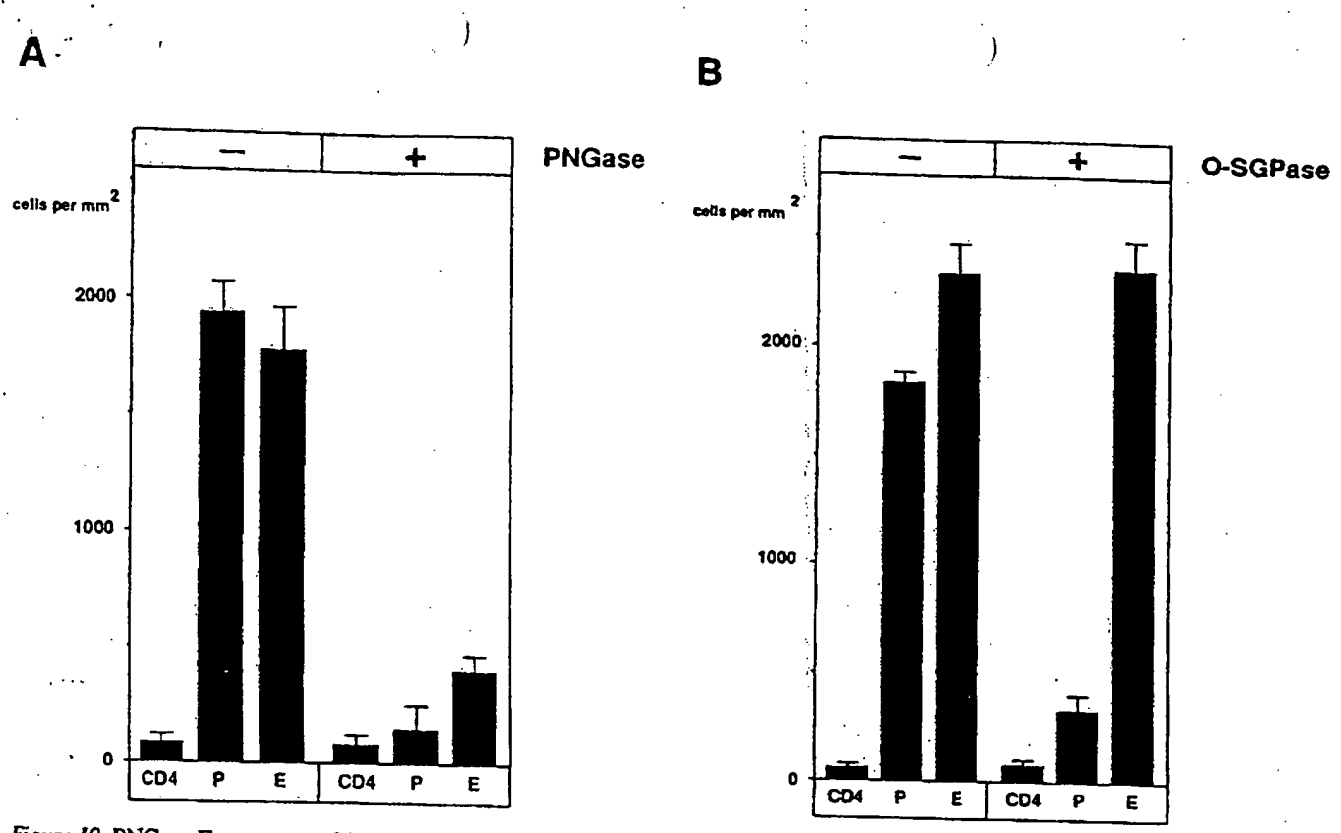

Figure 10. PNGase F-treatment of HL60 cells blocks binding to E- and P-selectin, while O-sialoglycoprotease-treatment only blocks cell binding to P-selectin. Cell adhesion assays were performed with HL60 cells in 96 well microtiter plates coated with CD4-IgG, P-selectin-IgG, or E-selectin-IgG (as indicated by *CD4, P, E*). Before the assay, cells were incubated with PNGase F (panel *A*, +), with O-sialoglycoprotease (panel *B*, +), or in the respective incubation buffers without the enzymes (panels *A* and *B*, −). Bound cells were quantitated by counting the cells under the microscope in 10 randomly chosen areas of defined size (per well) in four different wells for each determination. The depicted experiment represents one of three similar experiments. Bound cells were quantitated by counting the cells under the microscope in 10 randomly chosen areas of defined size (per well) in four different wells for each determination. The depicted experiment represents one of three similar experiments.

with O-sialoglycoprotease only blocked the binding of cells to P-selectin-IgG but not to E-selectin-IgG (Fig. 10 *B*). This is in agreement with the sensitivity of the P-selectin ligand and the resistance of the E-selectin ligand to this enzyme. We conclude that both monospecific selectin ligands are good candidates for cell adhesion ligands of P- and E-selectin.

Discussion

In this study we describe a 160-kD glycoprotein ligand for P-selectin on two myeloid cell lines and mouse neutrophils. This protein is clearly distinct from the recently identified 150-kD E-selectin ligand which is also present on these cells. Each of the two ligands is specific for only one of the two endothelial selectins. In addition to these ligands, two more weakly detectable glycoproteins of 230 and 130 kD were found, which bind to both endothelial selectins.

The 160-kD P-selectin ligand and the 150-kD E-selectin ligand fulfill the requirements for specific selectin ligands since they bind in a Ca^{2+}-dependent fashion, binding requires the presence of sialic acid on the ligands and no binding to the CD4-IgG control protein was observed. Since both ligands are specific for only one of the two endothelial selectins, the structural entities on each ligand which are recognized by the respective selectin must be different. This implies that ligand-binding sites on E- and P-selectin can be selective for different structural elements. Furthermore, both monospecific ligands require N-linked carbohydrate side chains for binding to the respective selectin. Although this demonstrates the lectin-type character of the binding mechanisms, the necessity of N-linked carbohydrates for binding clearly distinguishes this type of selectin-ligand-interaction from the one which was described for sialomucin-type selectin-ligands like the L-selectin ligands GlyCAM-1 and CD34. They are heavily O-glycosylated mucins which require sialic acid for the binding to L-selectin. Also, the 120-kD ligand for human P-selectin which was identified on human neutrophils and HL60 cells (Moore et al., 1992) is heavily sialylated on O-linked oligosaccharides and binds in a sialic acid dependent fashion (Moore et al., 1992; Norgard et al., 1993). The two monospecific 150-kD and 160-kD selectin ligands represent the first examples of glycoprotein ligands that require N-linked oligosaccharides for selectin-binding.

In addition to the two monospecific ligands for E- and P-selectin, we have found two glycoproteins of 230 and 130 kD which bind to both selectins in a Ca^{2+}-dependent fashion and do not bind to CD4-IgG. Both proteins were heavily sialylated, as judged from the decrease in electrophoretic mobility caused by sialidase treatment, and binding to the

selectins was dependent on the presence of sialic acid. In contrast to the two monospecific ligands, the 230 and 130-kD ligands were only weakly detectable (15–20-fold weaker signals) and could only be isolated from mouse neutrophils and not from the cell lines 32D cl 3 and HL60 (obtained from Amer. Type Culture Collection, see below). The weak detectability does not necessarily imply a low abundance of these proteins on neutrophils. Other reasons for the weakness of the signals could be a low turnover rate of the ligands (labeling was only done for 4 h) or a lower affinity for the binding to the selectins.

It is likely that the 130-kD glycoprotein which we isolated from mouse neutrophils with both mouse selectin fusion proteins is the homolog of the human 120-kD P-selectin ligand (Moore et al., 1992). Both proteins have a similar apparent molecular weight, show a similar strong decrease in electrophoretic mobility after sialidase treatment, and require sialic acid for the binding to P-selectin. In addition, the 130-kD mouse ligand was susceptible to digestion with O-sialoglycoprotease as was reported for the human 120-kD P-selectin ligand (Norgard et al., 1993), which is characteristic for sialomucins. A 110-kD sialomucin ligand for human P-selectin (PSGL-1) was recently identified by expression cloning (Sako et al., 1993). Similar to the 120-kD ligand described by Moore et al. (1992), the 110-kD protein forms a disulfide-linked dimer.

Although we could not detect the 230- and 130-kD ligands from HL60 cells of ATCC-origin, we detected proteins of similar size on the HL60 cells which we obtained from Genetics Institute (not shown). These were the same cells from which PSGL-1 was cloned, indicating the possibility that the 230/130-kD ligand-pair may be identical with PSGL-1. Indeed, HL60 cells from ATCC do not express PSGL-1 (Trudi Veldman, Genetics Institute, personal communication).

The following arguments support the idea that the 150- and 160-kD monospecific ligands are good candidates for cell adhesion ligands of the two selectins: First, both ligands are the only ones which we could detect on 32 D cl 3 cells and HL60 cells (of ATCC origin). Second, the inhibitory effect of PNGase F and endo F on the binding of these cells to the selectins correlates with the necessity of N-linked carbohydrates on both ligands for selectin-binding. This is in line with results obtained by Larsen et al. (1992), who found that tunicamycin treatment of HL60 cells partially blocked the binding to P-selectin and E-selectin. Third, O-sialoglycoprotease inhibits the binding of HL60 cells to P-selectin and degrades the 160-kD P-selectin ligand, but does not affect the binding of HL60 cells to E-selectin and does not cleave the 150-kD E-selectin ligand. Similarly, Steininger et al. (1992) found that this protease blocks HL60 binding to P- but not to E-selectin.

The sensitivity of the 160-kD P-selectin ligand to O-sialoglycoprotease suggests that this protein is O-glycosylated. This does not necessarily imply that its O-linked carbohydrates are involved in the binding to P-selectin. Also other glycoproteins, such as CD44 or CD45, are sensitive to O-sialoglycoprotease (Sutherland et al., 1992), although they do not carry carbohydrates which support selectin-binding. However, at present we cannot exclude that O-linked carbohydrates on the 160-kD ligand may also be involved in P-selectin binding.

The selectin ligands which we have described in this report seem to belong to two different categories. The first category is formed by the two monospecific ligands which each bind to only one type of selectin. Both ligands require sialic acid and N-linked carbohydrate side chains for binding. This looks like a different type of interaction than the binding of selectins to sialomucin-type ligands like GlyCAM-1, CD34, or the human 120-kD ligand for P-selectin. The mouse 230- and 130-kD glycoproteins which we have found as ligands for both endothelial selectins belong to the second category of ligands. The sensitivity to O-sialoglycoprotease, the irrelevance of N-linked carbohydrates for selectin-binding and the similarities between the 130-kD mouse protein and the 120-kD human P-selectin-ligand (Moore et al., 1992) and PSGL-1 (Sako et al., 1993) suggest, that the 230- and 130-kD ligands are of the sialomucin-type. This type of ligand seems not to be monospecific for one single selectin. Also GlyCAM-1 can bind to L-selectin and E-selectin (Mebius and Watson, 1993; M. Steegmaier and D. Vestweber, unpublished observation). For the sialomucin-type selectin ligands, it has been suggested that the selectins may recognize common O-linked oligosaccharides which are presented on such ligands as specific epitopes by forming unique "clustered saccharide patches" (Norgard et al., 1993). Such clusters are probably not present on the 150-kD E-selectin ligand, since it is resistant to O-sialoglycoprotease. It will be important to identify the structural motif on the two monospecific ligands which is recognized by each of the two selectins. The involvement of N-linked carbohydrates as well as the monospecificity for only one selectin argues for binding-sites on these ligands which are different from those of the typical sialomucin-type of ligands.

Whether the different types of ligands, which are coexpressed on neutrophils, reflect differences in their physiological role is still unknown. The common ligand(s) on neutrophils could allow these cells to use one and the same mechanism to bind to the two endothelial selectins. The monospecific ligands could provide the basis for different functions which the two selectins may exert on neutrophils when they bind to the blood vessel wall. Indeed, binding of E-selectin was reported to activate neutrophils (Lo et al., 1991; Kuijpers et al., 1991) while for soluble P-selectin, inhibitory effects on the CD11/CD18 dependent activation of neutrophils was reported (Gamble et al., 1990; Wong et al., 1991). Elucidation of the molecular function(s) of the identified E- and P-selectin ligands will be of central importance for the understanding of the physiological role of the endothelial selectins.

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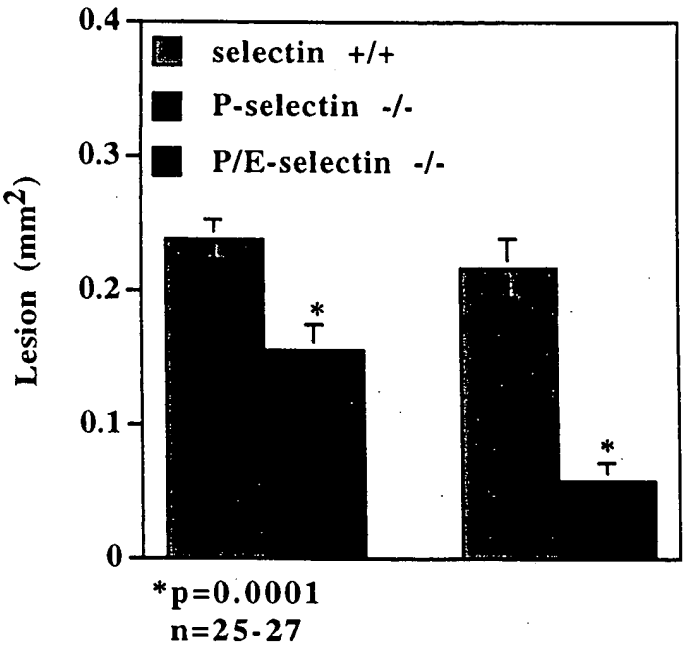
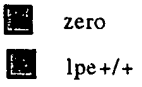
Aortic Sinus Lesions in LDL-Receptor -/- Mice on Atherogenic Diet for 8 Weeks

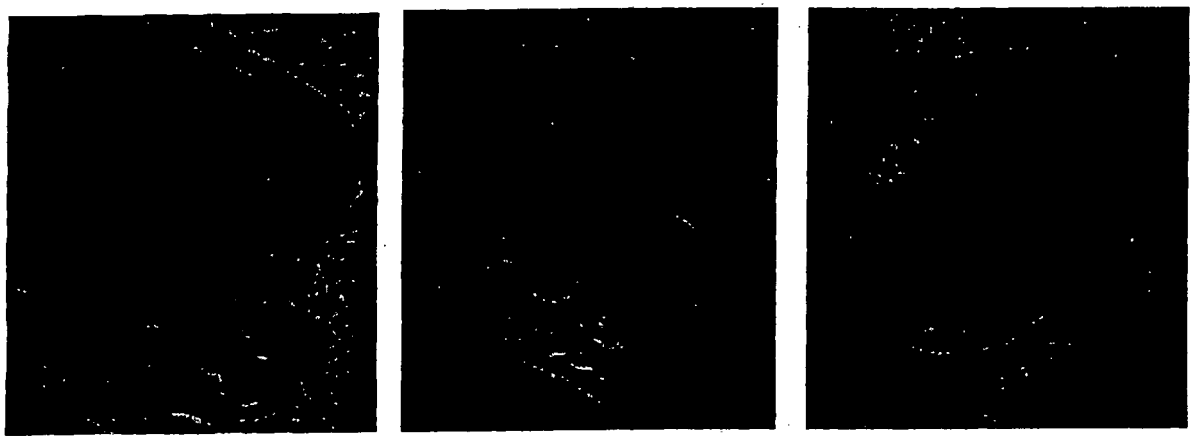




Atherosclerotic lesion in entire aortae of LDLR-deficient mice on diet for 22 or 37 weeks

	PE+/+	PE-/-	PE+/+	PE-/-
% area	39	19	51.6	37.7
	P < 0.006		P < 0.05	

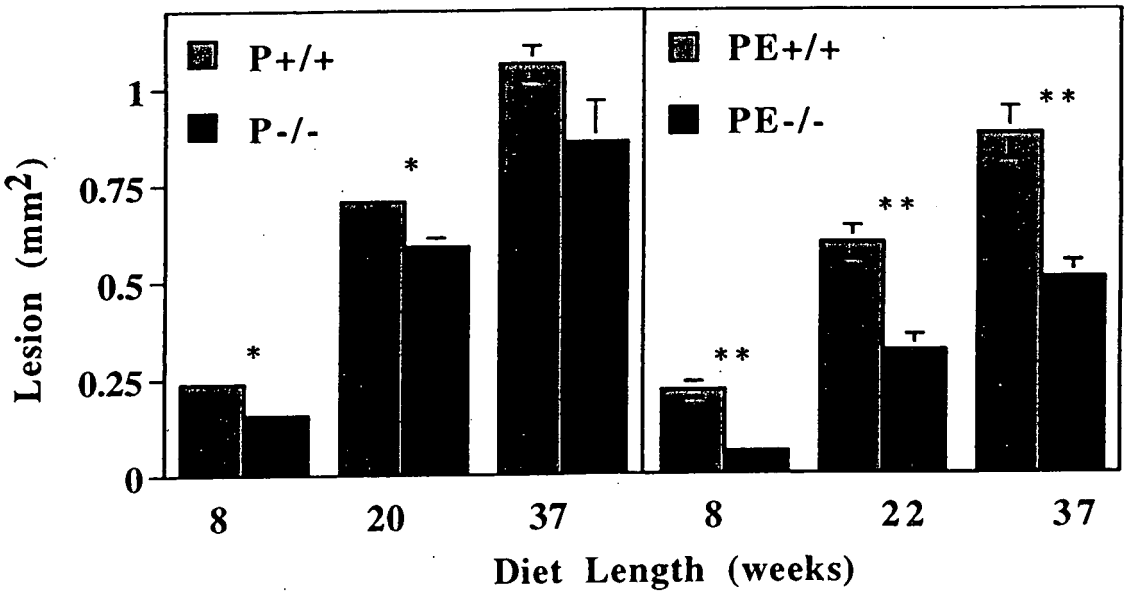
Aortic sinus lesions in LDLR-deficient mice on diet for 8 weeks



Wild type P -/- PE -/-

Fig. 3

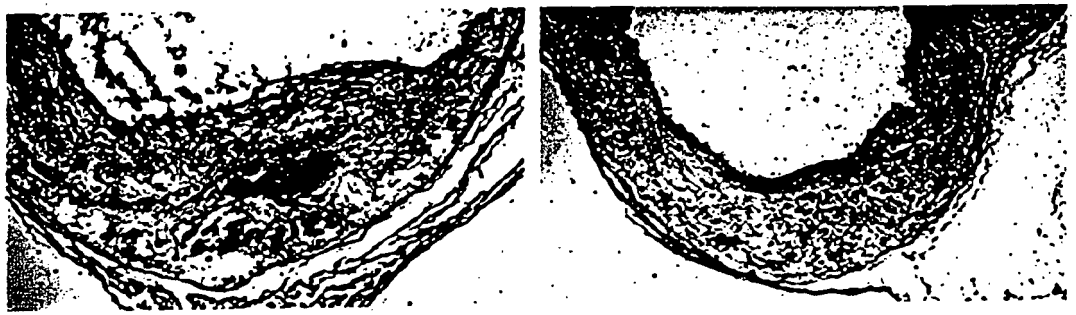
Atherosclerotic lesion development in the aortic sinus of LDLR-deficient mice



* P < 0.05, n = 10-27; ** P < 0.0005, n = 10-26

Fig. 4

Lesion calcification in the aortic sinus of LDLR-deficient mice on diet for 37 weeks



PE +/+

(93% of mice with calcification)

PE -/-

(20% of mice with calcification)

Lesions were stained with oil red 0, hematoxylin, and light green. Calcium deposit was identified by hematoxylin stain.

Fig. 5